THE BIOCHEMISTRY OF THE DEVELOPING CEREAL GRAIN

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ABBREVIATIONS

Standard abbreviations are used as in Biochem. J. 126 1972, pp. 1-19. These follow the Tentative rules and proposals of the IUPAC-IUB Commission on Biochemical Nomenclature (see Biochem. J. 101 1-7, 1966). Non standard abbreviations are introduced in the text, and are also listed below.

ADPG - ADP-glucose

ADPG* - ADP-glucose uniformly labelled with ^{14}C in the glucose moiety

CAMP - 3'5' cyclic adenosine monophosphate (cyclic AMP)

DCPIP - 2,6-dichlorophenolindophenol

F - fructose

F6P - fructose-6-phosphate

G - glucose

GIP - glucose-1-phosphate

G6P - glucose-6-phosphate

G*IP - glucose-1-phosphate uniformly labelled with ^{14}C

gm - gram

g - acceleration due to gravity

gr - grain

HEPES - N-2-hydroxyethylpiperazine-N-2-ethane sulphonic acid

Km - Michaelis constant

µgm - microgram

Rf - rate of movement of substance relative to solvent front

TCA - trichloroacetic acid

UDPG - UDP-glucose

UDPG* - UDP-glucose uniformly labelled with ^{14}C in the glucose moiety.

ENZYME NOMENCLATURE

The full name and enzyme commission number of all enzymes investigated are introduced in the text. All other enzymes mentioned in discussion or used in experimental techniques are listed below, together with the trivial name used in the text.

E.C.1.1.1.26	Glycollate:NAD oxidoreductase Glycollate:NADP oxidoreductase	glycoxylate reductase
E.C.1.1.1.37	L-Malate:NAD oxidoreductase L-Malate:NADP oxidoreductase	malate dehydrogenase
E.C.1.1.1.49	D-Glucose-6-phosphate:NADP oxidoreductase	glucose-6-phosphate dehydrogenase
E.C.1.2.1.12	D-Glyceraldehyde-3-phosphate: NAD oxidoreductase (Phosphorylating)	glyceraldehyde-3-phosphate NAD dehydrogenase
E.C.1.2.1.13	D-Glyceraldehyde-3-phosphate: NADP oxidoreductase (Phosphorylating)	glyceraldehyde-3-phosphate NADP dehydrogenase
E.C.3.1.3.11	D-Fructose-1,6-diphosphate 1-phosphohydrolase	fructose-1,6-diphosphatase
E.C.3.1.4.1	Orthophosphoric diester phosphohydrolase	cyclic phosphate diesterase
E.C.3.2.1.1	α-1,4-glucan 4-glucan hydrolase	α amylase
E.C.3.2.1.2	α-1,4-glucan maltohydrolase	β amylase
E.C.3.2.1.3	α-1,4-glucan glucohydrolase	glucoamylase
E.C.4.1.2.7	Ketose-1-phosphate aldehyde-lyase	aldolase
E.C.4.2.1.2	L-Malate hydro-lyase	fumarase

ABSTRACT

Concentrations of major biochemical constituents of developing barley endosperm were measured and correlated with morphological changes during maturation.

The activities of enzymes concerned with starch metabolism in barley endosperm were investigated. It was established that mechanisms by which sucrose can be converted to nucleotide sugars and thus incorporated into starch exist in the endosperm by 5 days after anthesis. Possible variations in these pathways during grain development are discussed.

The partition of starch synthetase between soluble and amyloplast fractions of the endosperm was established at various stages of development, and changes in affinity towards UDPG and ADPG were demonstrated. The relevance of these results to branching enzyme activity and amylopectin content is considered.

A detailed investigation of the activity and control of starch phosphorylase in barley endosperm was carried out. The existence of at least two isoenzymes was indicated by studies of pH dependence and phosphate inhibition, and was further supported by acrylamide gel electrophoresis and column chromatography using DEAE-cellulose. A change in the isoenzyme pattern during grain development was observed. Synthesis of starch by phosphorylase in an apparently primer-free system was demonstrated, and this activity integrated with starch synthetase.

A pathway for the conversion of sucrose to glucan polymers via GIP in very young endosperm is proposed. It is suggested

that at least one, and possibly both, isoenzymes of phosphorylase are glycoproteins. Spatial arrangements of phosphorylase, starch synthetase, and branching enzyme in relation to the amyloplast are discussed.

1. INTRODUCTION

Cereal grains have been recognised as a basis for foodstuffs since 3000 B.C.[1] They are of widespread economic importance in the present day, and have been studied in considerable detail. The germination stage of barley especially, which is of paramount interest to the brewing industry, has been the basis of much investigation. Much less is known about the biochemical changes taking place during the development of the grain, from anthesis until maturation. For this reason, the work described here is confined to these developmental stages of the barley grain.

1.1 MORPHOLOGICAL DEVELOPMENT OF THE CEREAL GRAIN

Fertilisation in the cereal flower involves the two male gametes from a pollen grain, one of which fuses with the ovum to form the embryo, while the other fuses with the two polar (or endosperm) nuclei, giving rise to the initial triploid endosperm nucleus.[2] This nucleus divides rapidly so that by 1 to 2 days after anthesis the embryo sac contains the zygote and a mass of free endosperm nuclei.[3] The embryo sac becomes lined with a single layer of nuclei, which later becomes the aleurone layer. Cell walls then begin to form between the nuclei, and the endosperm assumes a normal cellular structure.

Buttrose has published a series of electron micrographs of *Hordeum vulgare*[4] (barley) and *Triticum vulgare*[5] (wheat); these showed that the early endosperm cells contained

proplastids, mitochondria, and elements of the endoplasmic reticulum and Golgi apparatus. The proplastids could contain starch, but where they did not, they were difficult to distinguish from mitochondria. The normal double membrane of the proplastids was invaginated to form an internal structure of tubular processes; these tended to lie parallel to the outer membrane, in contrast to those of mitochondria, which were at right angles to the membrane. The starch granule lay in the plastid stroma, and rapidly increased in size until it occupied most of the stroma, obliterating the internal structures. This organelle could then be easily recognised as an amyloplast. About two to three weeks after fertilisation Buttrose observed small starch granules appearing in the plastid stroma, immediately below the membrane; these were released into the cytoplasm by extrusion of the membrane. Thus at maturity the endosperm contains two amyloplast populations; large plastids, 12 to 15 μm in diameter in barley, and small plastids, 2 to 3 μm diameter. Both wheat and barley contain amyloplasts which are oblate spheroids; they therefore appear either oval or spherical in cross section.

Buttrose also observed protein bodies in endosperm cells from 7 days after anthesis. Each granule was enclosed within a phospholipid membrane, often associated with the Golgi apparatus. The extent of protein accumulation is limited in barley, but wheat endosperm contains an average of 15% of its dry weight as protein.

The zygote develops into the embryo, which is situated at the base of the grain, and occupies only a small part of the mature grain. It is attached to a flat, shield-like structure, the scutellum, through which glucose is absorbed from the endosperm during germination, and transported to the growing embryo.[6]

The endosperm, aleurone and embryo are surrounded by the testa, a two layered integument forming the seed coat; the pericarp or ovary wall, which is composed of several layers of cells, and the husk, formed from the lemma and palea.[7] The two inner layers of the pericarp are chlorophyll bearing, so that the young grain appears pale green in colour, changing to a brighter green by 18 to 20 days after anthesis, when some of the outer layers of the pericarp are beginning to be reabsorbed, allowing the chlorophyll layer to show through. This resorption is greater at the base of the grain, in the region of the embryo. From 35 to 40 days after anthesis the grain begins to lose water, and the chlorophyll layer is reabsorbed: the testa and pericarp become very closely associated with the endosperm. The final stages of ripening are characterised by rapid desiccation of the grain and yellowing of the ear.

During the maturation process proteins, lipids, and especially starch, accumulate in the endosperm cells.[8] Cytoplasm is reduced to a thin lining along the inner surfaces of the plasma membranes, and the central vacuole so formed gradually fills with amyloplasts.[2] In the final stages of

maturation the cell structure is partially obliterated, and the cell contents - cytoplasm and nuclei - disappear. The outer layer of the endosperm, the aleurone, forms a membraneous sheath of rather regular cuboidal cells. These remain free from starch[7] and retain a secretory function in the germinating grain.[9,10] In wheat the aleurone cells can be distinguished by the presence of many "aleurone grains" around the nucleus; these grains consist of a core of soluble protein surrounded by a thin membrane. The cytoplasm of both wheat and barley aleurone cells is somewhat waxy or oily in nature.[7]

1.2 BIOCHEMICAL CHANGES IN THE CEREAL GRAIN DURING DEVELOPMENT

Jennings and Morton[8] surveyed the gross changes in carbohydrate, protein, lipid and water content during development of the wheat grain. They found that in the endosperm, macromolecular material such as starch increased both in relative and absolute concentrations. Low molecular-weight components such as reducing sugars and sucrose also provided a significant contribution to the dry weight of the endosperm in young grains; the amounts per grain increased gradually, but their proportion of the total dry weight declined abruptly during the period of rapid starch synthesis, as the precursor pool was depleted. The protein content, which increased in absolute concentration, also declined as a percentage of the dry weight, due to the increasing proportion of starch. Water content increased to a plateau around 20 to 30 days, then fell rapidly as desiccation commenced.

The changes in activity of many endosperm enzymes

involved in basic metabolism follow in outline the changes in water content. Marré,[11] who reviewed enyzmic activity in Ricinus communis (castor oil bean) during seed maturation, observed that the activity of enzymes such as aldolase, fructose-1,6-diphosphatase and malate dehydrogenase increased rapidly during development and attained maximum activity at about the same time as maximum fresh weight was achieved. Activity then declined to the low levels characteristic of the mature seed. Duffus[12] measured the activity of the endosperm enzymes during seed development in barley. She found a rapid increase in the carbohydrate metabolising enzymes glyceraldehyde-3-phosphate NAD and NADP dehydrogenases during the period in which proplastids develop into starchy amyloplasts. The decay of internal structure of the proplastids coincided with a sharp increase in catalase and peroxidase enzymes. Maximum activity of glyoxalate NAD and NADP reductase and malate NAD and NADP dehydrogenase was also noted around this period; these enzymes are probably concerned with lipid metabolism.[13] The lipid content of wheat[8] increases during the first month of seed development, mainly due to the proliferation of intracellular membranes.[5]

Mitochondrial activity, as exemplified by fumarase, appeared to decrease during development both in the castor bean[11] and barley endosperm.[12] This can be correlated with a requirement of a supply of reduced nucleotides for both lipid and carbohydrate synthesis.

Although they are rapidly synthesising macromolecular polymers, developing barley grains also contain active

hydrolytic enzymes. May and Buttrose[14] found that the production of maltose from a starch substrate rose to a maximum around 30 days after anthesis, the period of most rapid starch synthesis. This is mostly attributable to β amylase, but α amylase is also present.[15,16] Duffus and Rosie[17] demonstrated that soluble hydrolytic activity reached a maximum around 21 days in barley endosperm, and 29 days in the aleurone layer, subsequently declining in both fractions to low levels in the mature grain. Conversely, bound hydrolytic activity, which could be solubilised by papain treatment, remained high even in the mature grain. This may provide the source of some of the hydrolytic enzymes released during germination, although α amylase is synthesised de novo by the aleurone layer.[9] However, the rôle of these hydrolytic enzymes in the developing endosperm remains obscure.

1.3 THE SYNTHESIS OF STARCH

Starch and glycogen biosynthesis in plant[18,19] and animal[20] tissues was originally attributed to the enzyme phosphorylase which catalyses the reaction between glucose-1-phosphate (GIP) and glycogen or starch.

$$GIP + [G]_n \rightleftharpoons Pi + [G]_{n+1}$$

However in 1957 Leloir and Cardini detected an enzyme (UDP glucose:α-1,4 glucan α-4-glucosyl transferase, or starch synthetase) in liver tissue which could transfer glucose moieties to glycogen using a nucleotide sugar as a glucosyl carrier.[21]

$$\text{UDP-glucose} + [G]_n \longrightarrow UDP + [G]_{n+1}$$

A similar enzyme was later found to be associated with the starch granules of Phaseolus vulgaris (kidney bean).[22] The equilibrium constant for the transglucosylation reaction (Keq = 250 at pH 7.2) is much higher than for the phosphorylase reaction (Keq = 3 at pH 7.2); also the free energy of hydrolysis of the glycosidic phosphate bond in UDP-glucose (UDPG) exceeds that of the phosphate ester linkage in GIP.[23] These thermodynamic data, together with evidence of a starch-deficient mutant which lacks the enzymes necessary for nucleotide sugar synthesis[24] imply that glycogen and starch are synthesised predominantly by transglucosylase enzymes, using nucleotide sugars as carriers. In 1961 Recondo and Leloir[25] observed that starch synthesis in higher plants occurred faster with ADP-glucose (ADPG), although at that time ADPG was not recognised as a normal component of plant tissues. Subsequently ADPG was isolated from Zea mays (maize)[26] and Oryza sativa (rice) grains[27,28] and the ADPG-starch synthetase was found to be widely distributed in higher plants.[29] Jenner,[30] who measured levels of soluble nucleotides in developing wheat grains, noted that although the concentration of UDPG in the endosperm was greater than that of ADPG during the period of maximal starch synthesis, total concentration of adenine nucleotides exceeded uridine nucleotides. Since the rate of starch synthesis was 7 times faster with ADPG, and since also the rate of UDPG transglucosylase activity was considerably reduced by adenine nucleotides, whereas activity with ADPG remained unaffected by uridine nucleotides,[25] Jenner concluded that the major proportion of starch synthesised by

the wheat endosperm was formed via ADPG transglucosylase, although a small contribution by the UDPG enzyme could not be excluded.

The high levels of UDPG in starch synthesising tissues are considered by many workers to be associated with utilization of sucrose. Delmar and Albersheim,[31] working with bean seedlings, found that sucrose phosphatase and sucrose phosphate synthetase could only be detected in photosynthetic tissues, while sucrose synthetase occurred only in non-photosynthetic tissues. They suggested that the first two enzymes were concerned with sucrose synthesis from primary photosynthetic products, whereas sucrose synthetase (sucrose UDP glucosyl transferase) was associated with UDPG synthesis. Thus the glycosidic bond could be conserved, rather than being split by invertase activity. UDPG could be converted to GIP and UTP by UDPG pyrophosphorylase, which is generally very active in starch synthesising tissues. De Fekete and Cardini[32] proposed a similar mechanism, pointing out that the GIP could then be utilised for ADPG synthesis. Sucrose-UDP glucosyl transferase has been shown to exert a greater affinity for UDP than for ADP.[32,33] Sucrose synthesis by this enzyme is inhibited by UDP, but both UDPG and ADPG are equally effective as substrates.

The mechanism of starch biosynthesis however, remains incomplete. Both UDPG and ADPG starch synthetases require a glucan primer.[21,22,25,34] Leloir et al.,[35] working with a particulate starch synthetase from developing mung bean seeds ((P. aurens)) found that the addition of maltosaccharides

to the *in vitro* system decreased incorporation of labelled glucose into starch, longer chain maltosaccharides being preferentially synthesised. This suggests that transglucosylases are predominantly chain lengthening enzymes, and that another protein may be involved in the synthesis of short chain glucans. Slabnik and Frydman detected in young potato tubers (*Solanum tuberosum*) an isoenzyme of phosphorylase apparently capable of synthesising starch from GIP in the absence of added primer.[36] Tsai and Nelson[37] isolated two phosphorylase isoenzymes from developing maize endosperms one of which (designated Phosphorylase II) could utilize maltose as a primer, and also synthesise starch in a 'primer free' system. Furthermore, synthesis of a glucan polymer from GIP in the absence of added primer, and the incorporation of this polymer into starch by transglucosylase enzymes from destarched chloroplasts was demonstrated by Bird.[38]

The starch of mature barley contains on an average only 20% amylose,[39] consequently the remaining components cannot be entirely neglected. Branch points, α 1-6 linkages, are introduced into straight chain amylose by the so-called 'Q' or branching enzyme, (α-1,4 glucan:6glycosyl transferase).[40] Borovsky and Whelan[41] showed that in potatoes this enzyme required at least 50 glucose residues in both donor and acceptor amylose chains. In immature barley grains, the blue value of the starch was found in increase during development,[42,43] suggesting that amylopectin is the first product of starch synthesising enzymes, and that amylose only begins to accumulate as the grains approach maturity. The understanding of

mechanisms controlling this amylose/amylopectin ratio remains incomplete.

The experimental work described in the following pages is concerned mainly with starch synthesis in immature barley grains. Enzymes controlling the availability of key intermediates such as GIP and nucleotide sugars are investigated, and the existence and possible means of synthesis of glucan primers are also discussed.

2. EXPERIMENTAL SECTION. PART 1

The overall pattern of development in barley grain was first established by following the changes occurring in the major biochemical constituents of the endosperm throughout the period of maturation. Possible mechanisms of starch biosynthesis in developing endosperm were then investigated by confirming the presence and relative activities in this tissue of some of the enzymes which could be involved.

The section is divided into two parts:

2.A. Materials and methods.

2.B. Results.

2.A. MATERIALS AND METHODS

2.A.1. Plant material

The two row barley Hordeum distichum (L) Lam. c.v. Maris Baldric was used throughout the series of experiments. Plants were grown either in field plots or under greenhouse conditions as specified by Merritt and Walker.[43] Ears could be stored at -15°C for periods of up to 3 months without loss of activity for most of the enzymes assayed, although for some experiments on very young endosperm freshly gathered grains were used. The date of anthesis was determined by the system of Merritt and Walker,[43] but it was found that wide variations in the morphological stage of the grain were encountered when this system was used to evaluate the number of days after anthesis. A standardised system was therefore introduced, based on the average size of field barley. Thus the morphological condition of the grain, the size of the amyloplasts when viewed under the microscope, as well as the length of the awns, were taken into account when determining the number of days after anthesis.

2.A.2. Endosperm extracts

The husk and testa-pericarp were removed by hand and the remaining endosperm (including the aleurone layer) together with the embryo constituted the 'whole grain' subsequently referred to. This must be distinguished from the whole grain of several other workers (for example, Jennings and Morton[8]) which includes the testa-pericarp.

To prepare endosperm extracts, the whole grain was suspended in the appropriate buffer at 4°C and homogenised by

hand in an all-glass homogeniser. The homogenate was filtered through muslin to remove cell debris and centrifuged at 4°C for 10 minutes at 10,000 x g in a swingout head. The centrifuge used in all cases (unless otherwise specified) was an MSE High Speed 18. The supernatant solution which was pipetted off formed the soluble endosperm extract. The pelleted material was washed once with buffer, recentrifuged, and resuspended in buffer. This constituted the insoluble endosperm fraction. Microscopic examination of this fraction using a Vickers 15C light microscope equipped with phase contrast lenses (magnification x 1000) revealed that it was composed mainly of amyloplasts, and is subsequently referred to as the amyloplast fraction. Extracts from younger grain also contained small unidentified bodies 1μm in diameter; these were probably proplastids and mitochondria. The amyloplasts could be identified by staining with a 2% KI - 0.2% I_2 solution. The number of grains was varied from 60 (at 2-3 days) to 10 (18 days onwards) per 2 ml buffer.

2.A.3. Chemical analyses

Fresh weights were determined using a minimum of 10 grains. These were dried to constant weight at 108°C and reweighed to determine the water content and dry weight. Reducing sugars were estimated by the Somogyi-Nelson procedure,[44,45] and total carbohydrate by the anthrone reagent.[46] The difference between the total carbohydrate content of the whole endosperm, and that of the soluble endosperm extract gave a measure of the total insoluble carbohydrate content. This is mainly starch, together with some cell wall components. Soluble protein was measured by the Lowry method.[47] Amylose-

amylopectin ratios were calculated from the blue value,[48] following the procedure of Gilbert and Spragg.[49] Glucose was determined by a modification of the glucose oxidase method.[50]

Light dependent oxygen evolution in the chlorophyll containing layer of the testa pericarp at 25°C was demonstrated using a biological oxygen monitor (YSI Model 53). The testa pericarp fraction from 20 grains of 23 day barley was homogenised in Phosphate buffer (pH 7.2) containing 0.4M sucrose at 4°C. The homogenate was filtered through muslin and a 0.5ml sample of this solution used in a total assay volume of 3ml, containing 0.5ml of 2,6,Dichloro phenol indophenol (DCPIP) as an electron acceptor.[51] The reaction mixture was first flushed with nitrogen to eliminate dissolved oxygen.

2.A.4. Enzyme assays

Sucrose UDP glucosyl transferase (i.e. sucrose synthetase, E.C.2.4.1.13: UDP glucose; D-fructose 2-glucosyl transferase) was assayed by increase in reducing sugars after incubation of soluble endosperm extract with sucrose and UDP or ADP at 37°C.[52] Control samples without sucrose or without UDP(ADP) were incubated simultaneously. Samples were withdrawn for estimation of reducing sugars at 10 minute intervals over a period of 30 minutes. Since the rate of reaction was found to decrease after 20 minutes, initial reaction rates were used.

Invertase (β Fructofuranosidase E.C.3.2.1.26) was assayed by increase in reducing sugars after incubation of

the extract with sucrose at 37°C in Tris-Maleate buffer, pH 7.2, or in acetate buffer, pH 4.6.[53] EDTA (2 μmoles/assay) was added to the incubation mixture.

Starch Synthetase (UDP-glucose:α-1,4-glucan α-4-glucosyl transferase. E.C.2.4.1.11)

A modification of the method of Leloir et al.[35] was followed for estimation of starch synthetase in the amyloplast fraction. 0.1ml of this fraction was used as source of enzyme in a total reaction volume of 0.4ml. Control incubations contained water in place of UDP-glucose (UDPG) or ADP-glucose (ADPG). Samples were incubated for two hours at 37°C and the UDP or ADP released estimated colorimetrically.[54] Activity in the amyloplast and soluble endosperm fractions was compared by measuring the incorporation into starch of radioactively labelled glucose (^{14}C-glucose) from uridine-5'-diphospho(glucose-C^{14}(U)) (UDPG*) or from adenine-5'-diphospho (glucose-C^{14}(U)) (ADPG*).[35] The UDPG* (specific activity 233 mCi/m mol.) was obtained from the Radiochemical Centre, Amersham, and the ADPG* (specific activity 75 mCi/m mol.) was a gift from Dr. I.F. Bird, (Rothamsted Experimental Station).

The incubation mixture contained:-

0.025 μCi UDPG* or 0.0225 μCi ADPG*,

0.3 μmoles unlabelled UDPG or ADPG,

0.05ml 2.5% (w/v) soluble starch,

0.1ml endosperm fraction,

0.1ml EDTA (0.2mM)

0.2ml Glycine buffer, pH 8.3 (0.05M),

in a total volume of 0.525ml.

After incubating for 4 hours at 37°C, solid starch was added as a carrier and the mixture centrifuged for 10 minutes at 13,000 x g to collect the insoluble material. Unreacted UDPG* (or ADPG*) was removed by washing the pellet three times with distilled water, before resuspending in 0.5ml. distilled water by boiling gently for 5 minutes. Samples were counted for 50 minutes in 5ml of dioxan-based liquid scintillator (N.E.220. Nuclear Enterprises, Edinburgh) in a Beckmann Liquid Scintillator System. Samples were automatically corrected for background radiation. Counting efficiency for C^{14} was about 25%. Duplicate samples were run, and all values corrected for controls incubated without enzyme extract.

Branching enzyme. (α-1,4-glucan; α-1,4-glucan 6-glycosyl transferase. E.C.5.3.1.9).

Estimation of branching enzyme activity in barley endosperm is complicated by the presence of α and β amylases. Since amylopectin gives a less intense colour with iodine than does amylose, the activity of both amylases and branching enzyme will tend to diminish the intensity of colour a given amount of starch will form with iodine reagent. While amylases can utilise both glycogen and amylopectin as substrates at the same rate, branching enzyme can only act on amylopectin. The method of Krisman[55] for assaying branching enzyme exploits this difference in substrate specificity. The reduction of iodine colour during incubation of the endosperm extract with (a) glycogen, and (b) amylopectin is determined. The difference between these two values indicates branching activity in

the extract.

Results were corrected for control assays in which endosperm extract was added after incubation with the substrate. Absorption spectra of the amylopectin-iodide complex formed after incubation with branching enzyme was compared to equivalent spectra of amylopectin and glycogen alone.

Assays were carried out in sodium citrate buffer, pH 6.4, and activity was observed to be closely dependent on citrate concentration. A final concentration of 0.15M citrate in the reaction mixture was found to give the highest activities.

The supernatant solution (S24) obtained by centrifugation of the soluble endosperm extract for 1 hour at 24,000 x g appeared to have higher branching activity than the original extract. The results given (page 39) were obtained using this S24 extract, in a 0.15M citrate buffer.

ADPG and UDPG pyrophosphorylase, (UTP(ATP) α-D-glucose-1-phosphate uridylyl (adenyl) transferase. E.C.2.7.7.9.

These enzymes were assayed in the direction of ADPG (UDPG) breakdown, by coupling to the glucose-6-phosphate dehydrogenase reaction.[56] Phosphoglucomutase, glucose-6-phosphate dehydrogenase and $NADP^+$ were added to the reaction mixture to convert GIP formed by pyrophosphorylase activity to NADPH, the formation of which could be followed spectrophotometrically.

$$\text{UDPG (ADPG)} + \text{PPi} \rightleftharpoons \text{UTP (ATP)} + \text{GIP}$$

$$\text{GIP} \rightleftharpoons \text{G6P}$$

$$\text{G6P} + NADP^+ \rightleftharpoons \text{6-phospho gluconate} + \text{NADPH}.$$

Results were expressed as nMoles UDPG (ADPG) broken down/ minute/10 grains. In control assays pyrophosphate was replaced by orthophosphate.

Inorganic pyrophosphatase. (Pyrophosphate phosphohydrolase. E.C.3.6.1.1.)

Activity was estimated by the increase in inorganic phosphate after incubation for 15 minutes at 30°C with 1 µmole of sodium pyrophosphate in a total volume of 1ml, in 0.05M tris-Maleate buffer, pH 7.2.[57] Orthophosphate was measured by the method of Fiske and SubbaRow.[58]

Hexokinase. (ATP:D-Hexose-6-phospho transferase. E.C.2.7.1.1.)

This enzyme was detected by coupling the G6P formed to G6P dehydrogenase, as previously described (page 17),[56] except that phospho gluco mutase was omitted. Both glucose and fructose were employed as substrate,[53] and gave similar results, although the values shown were obtained with glucose. Controls without ATP, and without glucose, were subtracted from the results, which were expressed as n moles glucose removed/minute/grain.

Glucose-6-phosphate keto isomerase. (D-Glucose-6-phosphate ketol-isomerase. E.C.5.3.1.9.)

Activity was measured by incubating 0.1ml of endosperm extract with 4µmoles of G6P in the presence of 100µmoles of tris buffer pH 8.0.[53] The formation of fructose-6-phosphate (F6P) was measured by the resorcinol thio-urea method.[59]

Phosphoglucomutase (D-Glucose-1,6-diphosphate: D-glucose-1-phosphate phosphotransferase. E.C.2.7.5.1.)

This assay exploited the difference in susceptibility to hydrolysis between GIP and G6P.[53] GIP was incubated with endosperm extract in tris buffer pH 7.4, for 30 minutes at 30°C. After treatment with $5N_2H_2SO_4$, the unreacted GIP was hydrolysed to glucose and inorganic phosphate, while G6P remained unchanged. The original concentration of GIP was measured by adding the GIP to the reaction mixture after incubation, but before H_2SO_4 treatment. The amount of GIP converted to G6P was then given by the difference in concentrations of inorganic phosphate.[58]

Nucleoside di-phosphate kinase. (ATP: nucleoside di-phosphate phosphotransferase. E.C.2.7.4.6.)

Activity was estimated by incubating endosperm extract with ADP and UTP or GTP in N-2-hydroxyethylpiperazine-N-2-ethane sulphonic acid (HEPES) buffer, pH 7.5.[60] The ATP formed was coupled to the glucose-6-phosphate dehydrogenase reaction as previously described (page 17).[56] Glucose, hexokinase, $NADP^+$ and glucose-6-phosphate dehydrogenase were added to the reaction mixture.

$$ADP + UTP\ (GTP) \rightleftharpoons ATP + UDP\ (GDP)$$

$$ATP + G \rightleftharpoons G6P + ADP$$

$$G6P + NADP^+ \rightleftharpoons 6\ \text{phospho gluconate} + NADPH.$$

Results are expressed as n moles GTP(UTP) used/minute/grain.

2.B. RESULTS

2.B.1. Morphology

Changes in the main morphological features of the barley grain during development are summarised in Table 1. During the first two days after anthesis numerous organelles 1 μm in diameter were revealed by microscopic examination of endosperm tissue. These were most probably proplastids and promitochondria, but it was not possible to distinguish between them at this stage. Amyloplasts, 2 μm in diameter could be stained with iodine reagent by 7 days after anthesis. The endosperm at this stage formed a turgid, discrete body, easily dissected out of the grain.

Between 7 and 10 days after anthesis the endosperm elongated rapidly, while amyloplast size remained relatively unchanged, resulting in a very watery endosperm which was difficult to separate from the surrounding testa-pericarp. Fig. 1 is a diagrammatic representation of a barley grain at this stage of development with the glumes and lemma removed. Two anthers and the remains of the stigmas can be seen. The endosperm, covered by the testa pericarp, has elongated to occupy half of the space between the tip and the base of the palea.

A steady rate of growth, both in overall endosperm dimensions, and in amyloplast diameter, was maintained between 12 and 20 days after anthesis. Plate 1 shows a photomicrograph of the amyloplast fraction from 15 to 17 day grain, stained with iodine reagent.

By 20-22 days the amyloplasts were 9-10 μm in diameter.

TABLE 1. Changes in Main Morphological Features of Developing Barley Grain

Age in days after anthesis	Size of Amylo-plasts (μm)	Morphological description of grain	Colour of grain
2		Free nuclear stage	
3	1	Proplastids and Promito-chondria	
5	1	Formation of cell walls	Pale
7	2	Turgid 'pearl like' endo-sperm. Amyloplasts stain with iodine	Green
10-12	3-4	Watery endosperm	Bright green due to chloro-phyll layer of testa-pericarp
14	3-4	Thin sliver of endosperm 3-4 mm	
15	5	Endosperm thickening	
18	6	Milky stage of endosperm embryo can be separated	
21	8	aleurone can be scraped off endosperm	
25	10		
30	12	Chlorophyll layer begins to be reabsorbed	Turns yellow, begin-ning with awns
33-35	15-17		
40	16	Endosperm begins to dry out. Testa-pericarp adhering to endosperm	
45-50	15		
50-60	15-16	Grain shrinks due to water loss	

Figure 1. Diagrammatic representation of Barley grain 8 days after anthesis, with glumes and lemma removed. Scale. Approx. x 20.

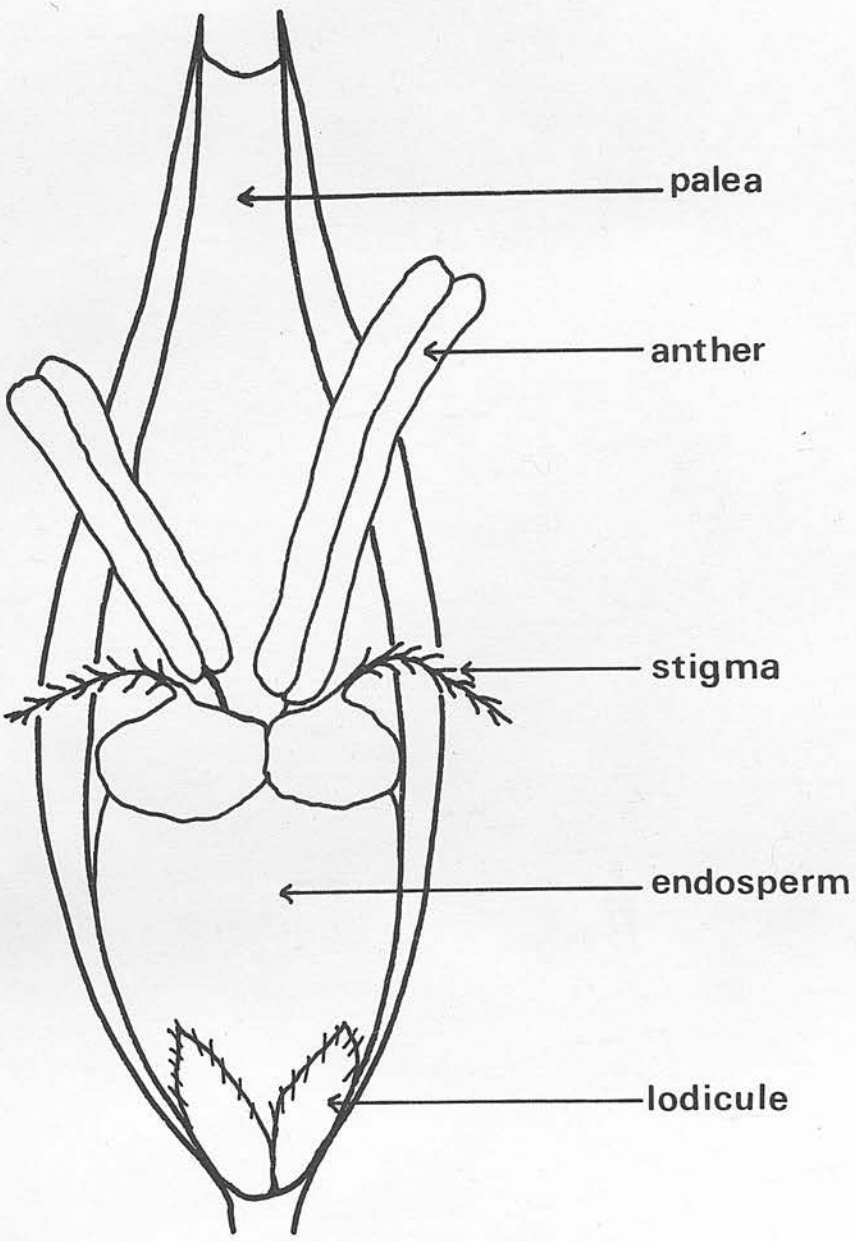
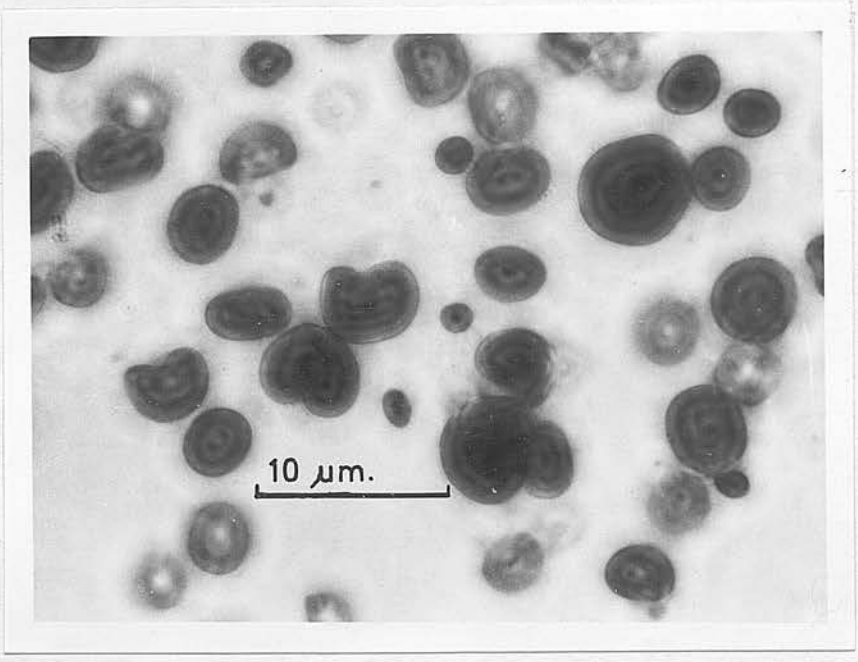

palea
anther
stigma
endosperm
lodicule

Plate 1. Photomicrograph of amyloplast fraction of 15 to 17 day endosperm. Stained with I_2-KI. Scale. 10μm as indicated.

1.



At this stage the embryo could be distinguished from the endosperm, and the aleurone layer could be removed by scraping the outside of the endosperm. Maximum endosperm dimensions and amyloplast size were achieved around 30 to 40 days after anthesis. Desiccation commenced in the awns, spreading to the grain and causing slight shrinking of the endosperm and of the amyloplasts in the final stages of maturity.

2.B.2. Chemical Analyses

The changes in fresh and dry weight, and water content, expressed in mg/grain, are shown in Fig. 2. Water content, and consequently fresh weight, increased rapidly from 7 days after anthesis, reaching a maximum around 40 days, and thereafter declining. Dry weight, however remained fairly low until 16 days, then rose steadily to a maximum around 44 days, and did not fall much below this value during further maturation. From Fig. 3 it can be seen that the percentage water content was highest in grain around 12 days after anthesis, and declined steadily after this stage, until in the mature grain less than 20% of water could be detected.

Figs. 4 and 5 indicate variation during development of total carbohydrate in the whole endosperm, and of total carbohydrate and reducing sugars in the soluble endosperm extract. The level of total carbohydrates remains fairly low until 14 to 15 days after anthesis, when synthesis commences, the most rapid rate of accumulation occurring between 28 and 36 days after anthesis. Carbohydrate content is maximal around 44 days; levels then decrease slightly. Although the concentration of soluble carbohydrates remains relatively low, a significant decline can be observed around 20 days, coinciding with the initial increase in total carbohydrates. Levels of reducing sugars remain low in young endosperm, gradually increasing around 20 days, to a broad maximum between 40 - 50 days.

Figure 2. Changes in fresh weight, dry weight and water content of whole grain.

fresh weight ●

dry weight o

water content ×

Figure 3. Water content of whole grain as a % of fresh weight.

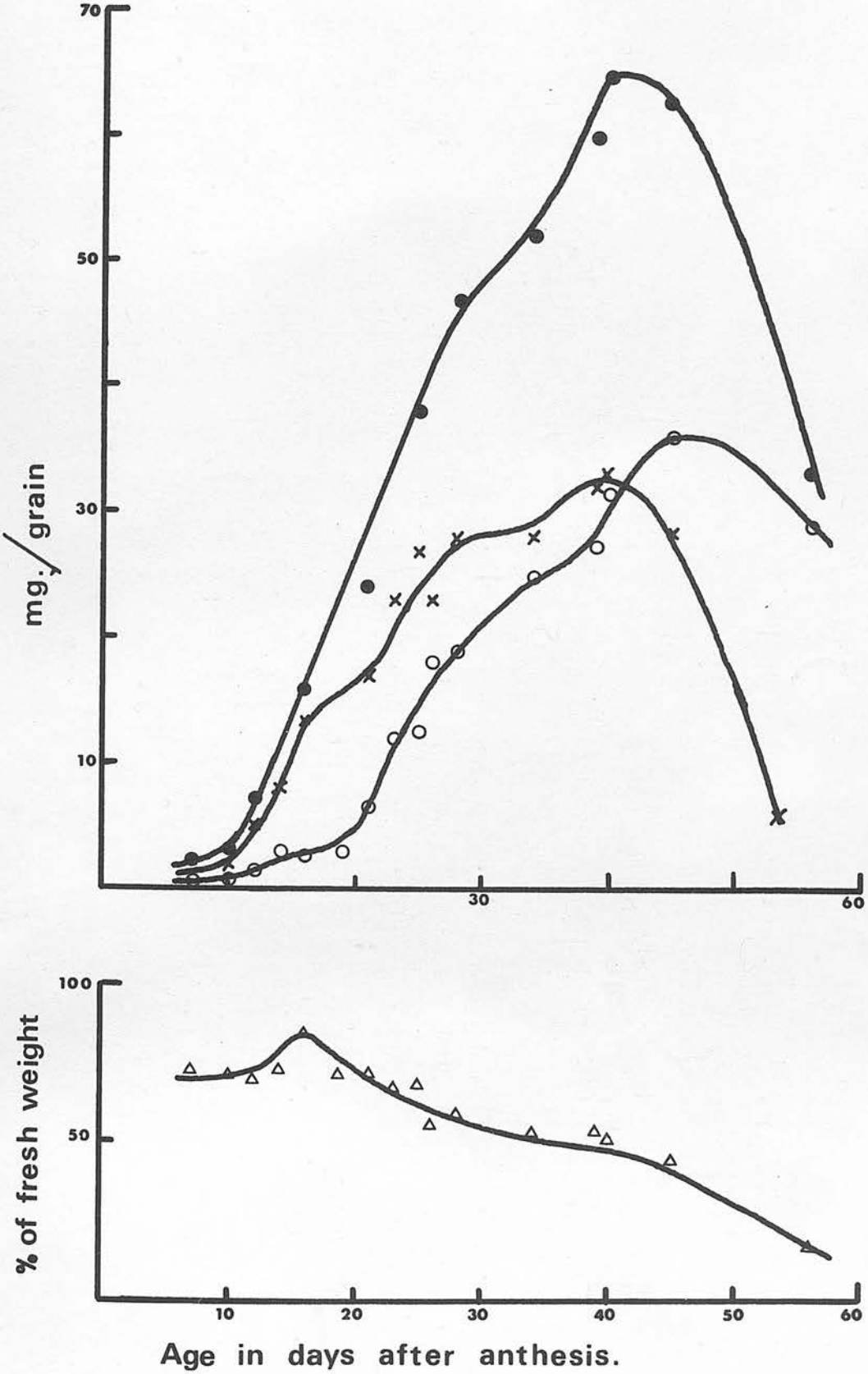
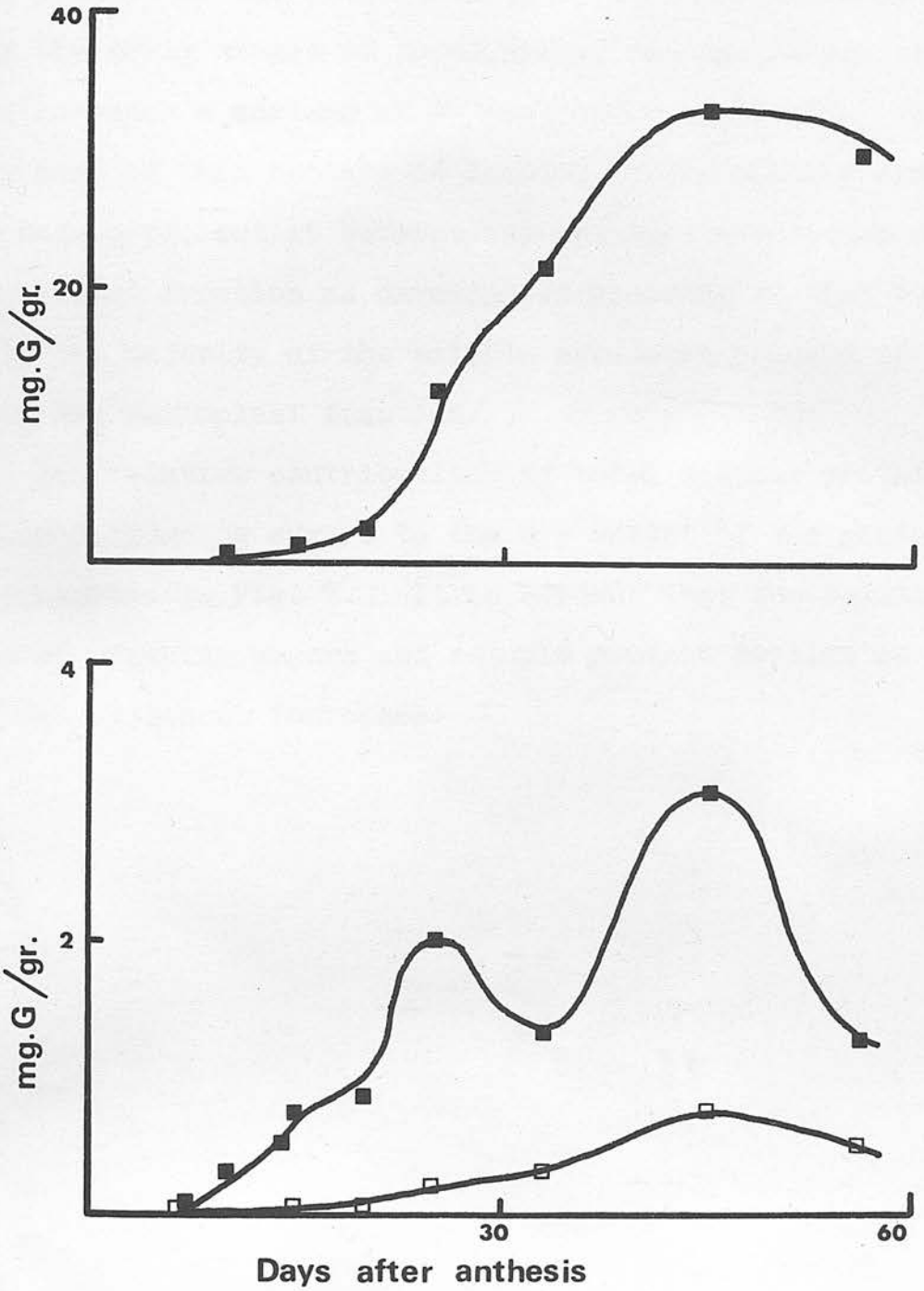

70
50
30
10
mg./grain
30
60
100
50
% of fresh weight
10
20
30
40
50
60
Age in days after anthesis.

Figure 4. Total carbohydrate content of whole grain, expressed as mg. glucose/grain.

Figure 5. Total carbohydrate and reducing sugar content of soluble endosperm extract, expressed as mg. glucose/grain.

total carbohydrate ■

reducing sugars □


40
20
mg.G/gr.
4
2
mg.G /gr.
30
60
Days after anthesis

Changes in soluble protein content of endosperm fractions are expressed in Fig. 6. The concentration of soluble protein in the whole endosperm increases steadily through the early stages of development, rising sharply at 28 days to reach a maximum at 44 days after synthesis. Initially most of this protein is located in the soluble fraction of the endosperm, but it becomes increasingly associated with the amyloplast fraction as development proceeds so that by maturity the majority of the soluble endosperm protein is found in the amyloplast fraction.

The relative contributions of total soluble protein, starch, and reducing sugars to the dry weight of the grain are represented in Fig. 7. It is evident that the relative amounts of reducing sugars and soluble protein decline as the proportion of starch increases.

Figure 6. Soluble protein content of endosperm.

whole grain ●

soluble endosperm extract o

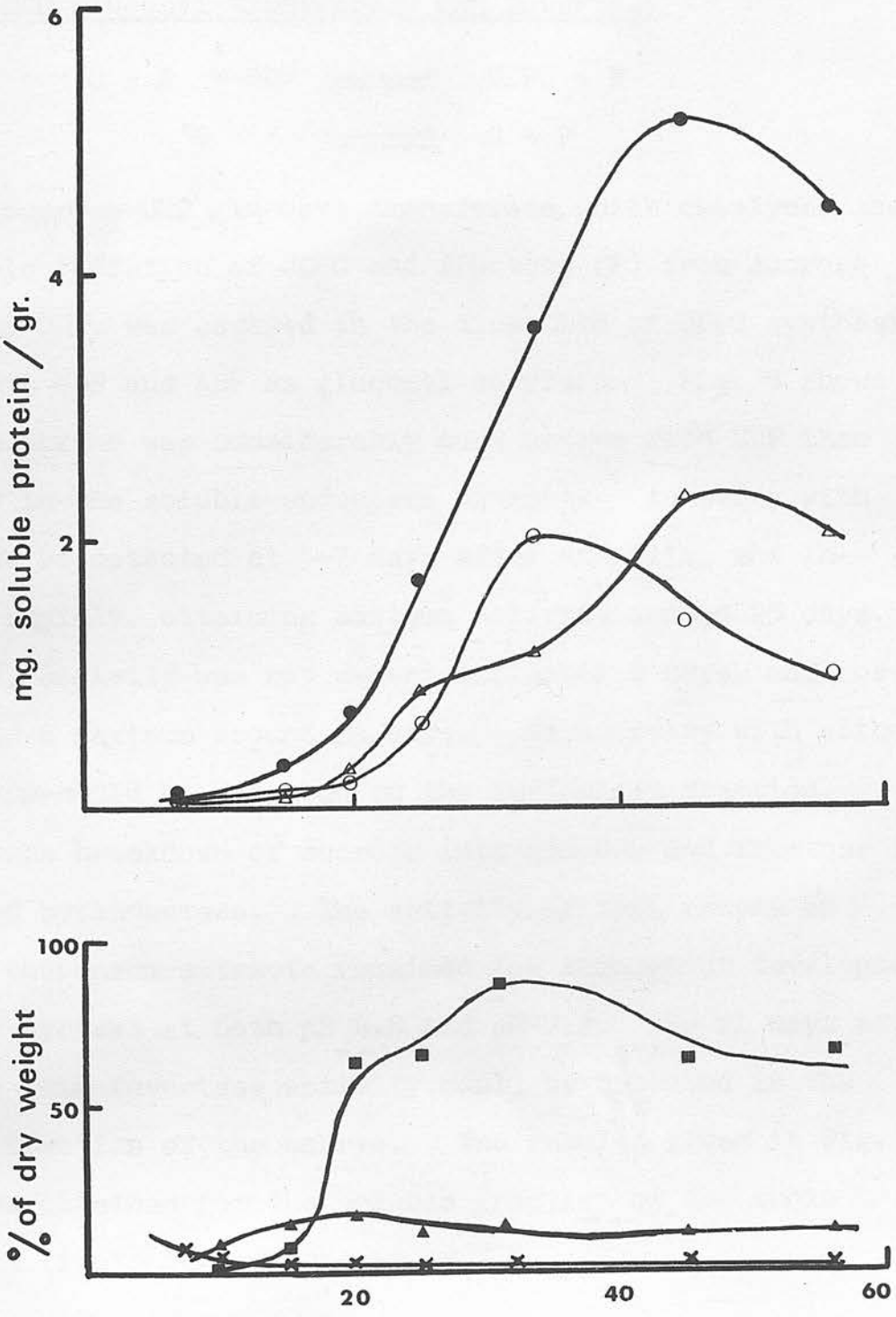
amyloplast fraction. Δ

Figure 7. Starch, reducing sugars and soluble protein as a % of dry weight.

starch ■

reducing sugars X

soluble protein of whole grain. ▲


6
4
2
mg. soluble protein / gr.
100
50
% of dry weight
20
40
60
Days after anthesis

2.B.3. Enzymology

Sucrose-UDP glucosyl transferase and Invertase

$$G - F + UDP \rightleftharpoons UDPG + F$$

$$G - F \rightleftharpoons G + F$$

Sucrose-UDP glucosyl transferase, which catalyses the reversible formation of UDPG and fructose (F) from sucrose (G-F) and UDP, was assayed in the direction of UDPG synthesis, using both UDP and ADP as glucosyl carriers. Fig. 8 shows that the enzyme was considerably more active with UDP than with ADP in the soluble endosperm extract. Activity with UDP could be detected at 5-7 days after anthesis, and increased rapidly, attaining maximum activity around 25 days. With ADP, activity was not detectable until 8 days, and rose slowly to a maximum around 34 days. No activity with either UDP or ADP could be detected in the amyloplast fraction.

The breakdown of sucrose into glucose and fructose is catalysed by invertase. The activity of this enzyme in soluble endosperm extracts remained low throughout development in assay systems at both pH 4.8 and pH 7.2. By 21 days after anthesis some invertase activity could be detected in the soluble fraction of the embryo. The results given in Fig. 9 are those obtained for the soluble fraction of the whole endosperm (including embryo) at pH 7.2.

Light dependent oxygen evolution

Fig. 9 indicates that a testa-pericarp homogenate from 23 day grains was capable of light dependent oxygen evolution using DCPIP as an electron acceptor. The rate of

Figure 8. Sucrose UDP glucosyl transferase and Invertase activity in soluble endosperm extracts.

● sucrose-UDP glucosyl transferase

o sucrose-ADP glucosyl transferase

X invertase.

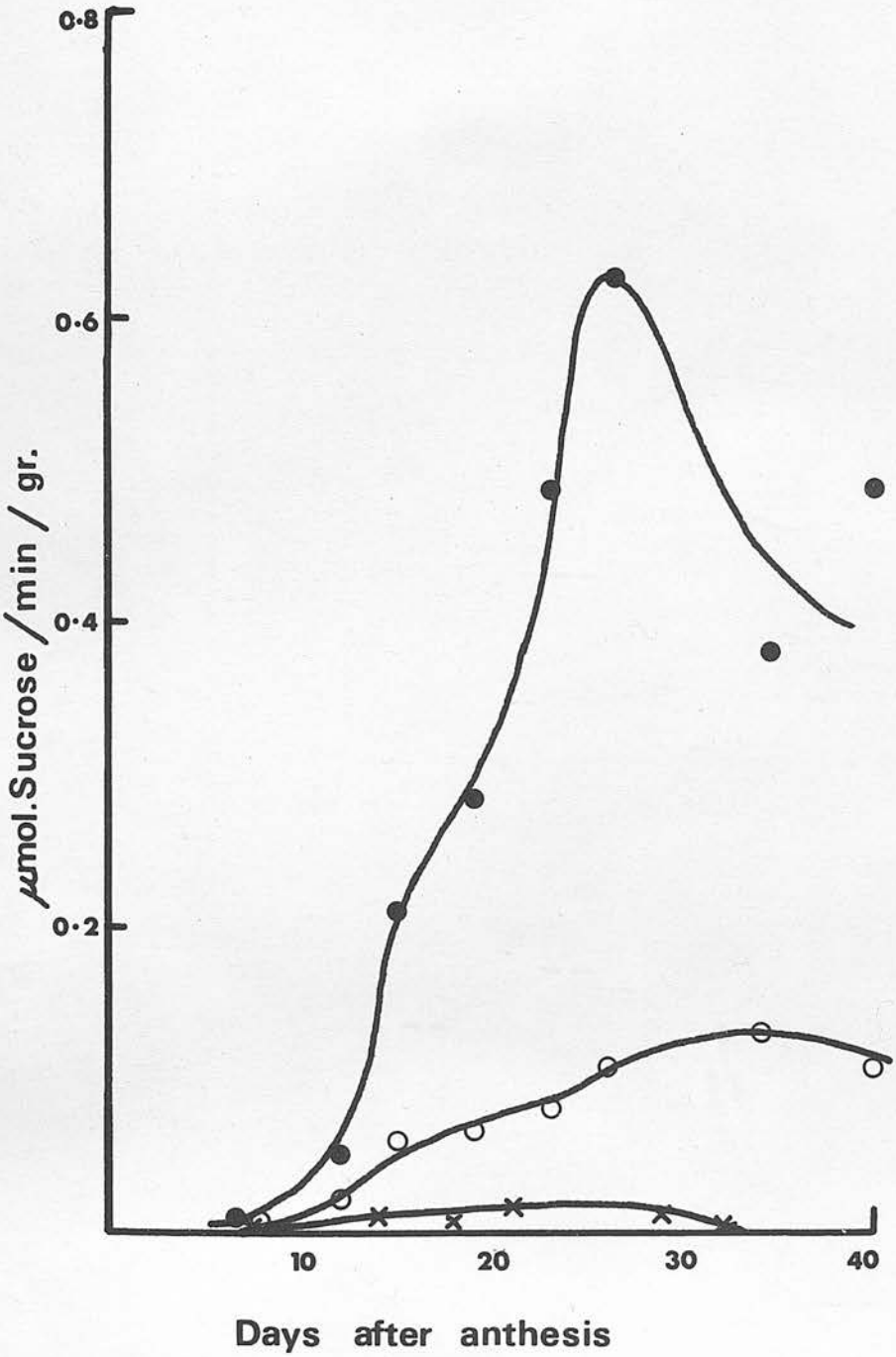
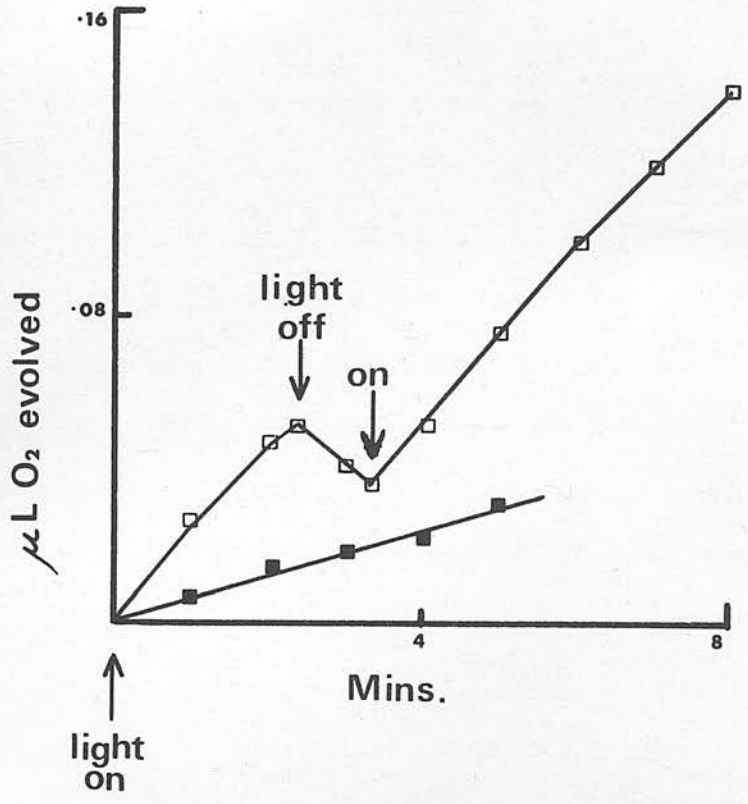

0·8
0·6
0·4
0·2
µmol.Sucrose /min / gr.
10
20
30
40
Days after anthesis

Figure 9. Light-dependent oxygen evolution by testa-pericarp fraction of 23 day grain.

□ illuminated sample

■ control sample wrapped in foil.


·16
·08
μL O2 evolved
light
off
on
4
8
Mins.
light
on

oxygen evolution was very much reduced in control samples shielded from the light by aluminium foil.

Starch Synthetase

$$G_{(n)} + \underset{(ADPG)}{UDPG} \longrightarrow G_{(n+1)} + \underset{(ADP)}{UDP}$$

This enzyme catalyses the transfer of glucose from UDPG and ADPG to a glucan primer, and exists in either a soluble form, or a bound form attached to starch granules. Both types of activity could be detected in barley endosperm. Figs. 10 and 11 show the variation in amyloplast bound activity during development, using either UDPG or ADPG as glucosyl carrier. UDPG-linked activity predominated in young grain, being detected as early as 6 days after anthesis. An abrupt increase was apparent between 14 and 18 days, slowing to an extended period of maximal activity between 20 and 25 days.

The ADPG enzyme could not be detected before 10 days after anthesis. Activity then rose rapidly between 12 and 20 days, maximal reaction rates being twice those observed with UDPG, but declining again by 23 days after anthesis.

Bound and soluble starch synthetase activities were compared by the extent of incorporation of ^{14}C-glucose from UDPG* or ADPG* into starch. Table 2 demonstrates that in young grain UDPG starch synthetase is predominantly soluble, but becomes progressively bound to the amyloplasts as development proceeds.

The data given in Table 3 imply that the bound enzyme has a very much greater affinity for ADPG than for UDPG, confirming the previous results. In the soluble fraction, however, activity with UDPG* exceeded that with ADPG*.

Figure 10. Starch synthetase activity in amyloplast fraction of developing grain.

with UDPG ●

with ADPG ○

Figure 11. Starch synthetase activity with UDPG in amyloplast fraction as % of total starch synthetase of that fraction.

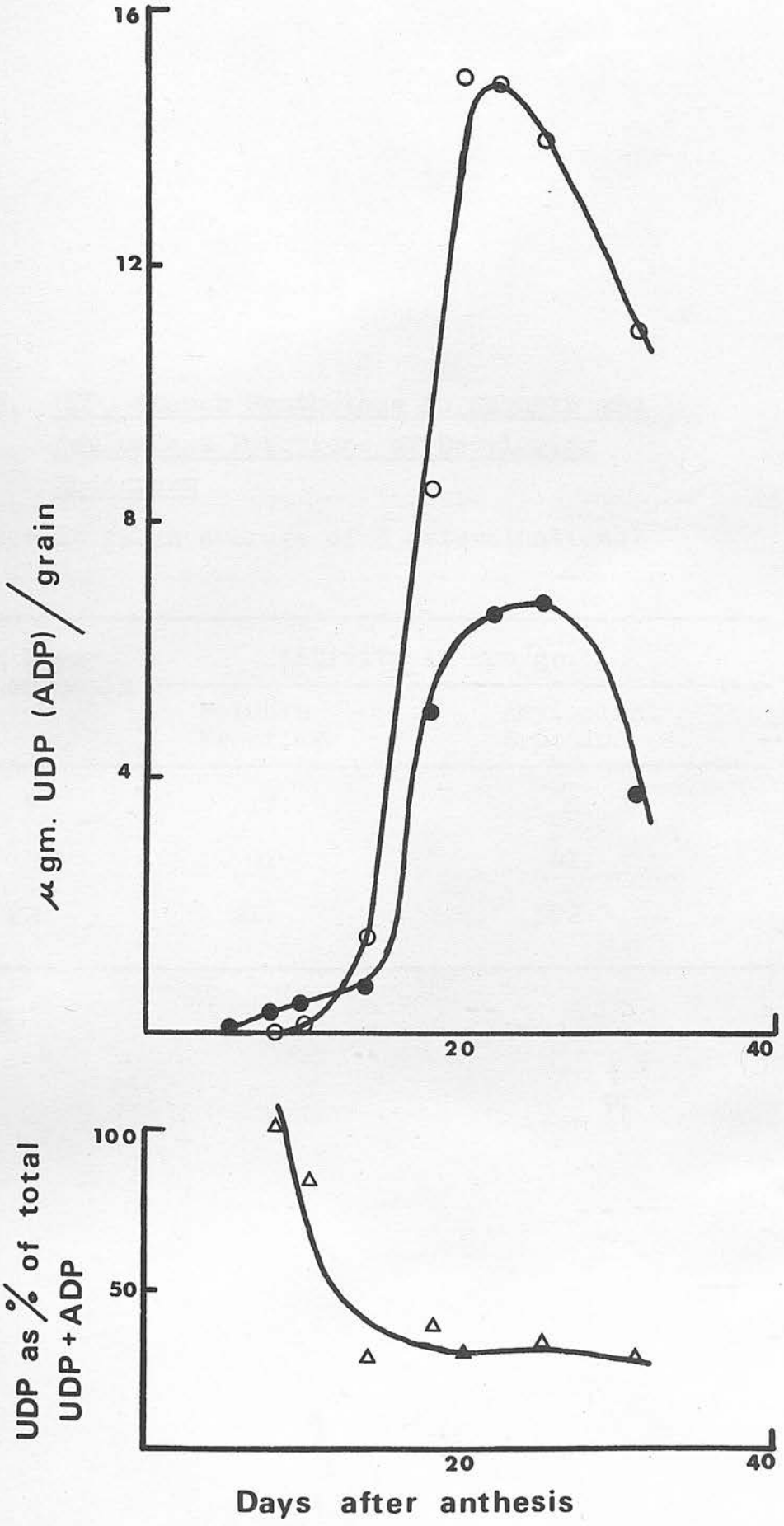

16
12
8
4
μgm. UDP (ADP) / grain
20
40
100
50
UDP as % of total
UDP + ADP
20
40
Days after anthesis

TABLE 2. UDPG-Starch Synthetase in Soluble and Amyloplast Fractions of Developing Endosperm

(Each result is an average of 3 determinations)

Age in Days after anthesis	Activity in cpm/gr.	
	Soluble Fraction	Amyloplast Fraction
7	17	8
18	91	41
22	217	592

TABLE 3. UDPG and ADPG as Glucosyl Donors for Starch Synthetase Activity in 20 day Endosperm Extracts

(Each result is an average of at least 4 determinations)

Glucosyl Donor	Activity in cpm/gr.	
	Soluble Fraction	Amyloplast Fraction
UDPG*	299	109
ADPG*	27	1273

Branching enzyme activity and Amylopectin content

Fig. 12 shows the percentage content of amylopectin in barley starch during development. It is evident that the percentage of amylopectin in young endosperm (15 days after anthesis) was very high - almost 90% - gradually declining to around 75% which is characteristic of mature barley starches.

Branching enzyme activity in the soluble endosperm S_{24} fraction (see page 17) could be detected as early as 3 days after anthesis, and increased rapidly, reaching a maximum fairly early in development, around 18 days, thereafter declining quickly (Fig. 13). Significant, although variable, branching activity was detected in the amyloplast fraction. This activity could be considerably increased by mechanical disruption of the amyloplasts by grinding in a mortar and pestle. The amyloplast activity depicted in Fig. 13 refers to the soluble extract obtained by centrifugation of this ground-up amyloplast fraction for 10 minutes at 5,000 x g.

The amyloplast bound activity was not detectable until 14 days after anthesis, but continued for some days after soluble activity had disappeared.

It is clear from Fig. 14 that incubation of amylopectin with soluble endosperm extract caused a shift in the absorption maximum of the amylopectin-iodide complex from 480 nm to 400 nm. This altered absorption spectrum closely resembled that of glycogen, confirming that branching, rather than amylolytic, activity was being measured.

Figure 12. Amylopectin content as % of total barley starch.

Figure 13. Branching enzyme activity in endosperm during development.

□ soluble fraction S_{24}

■ ground up amyloplast extract.

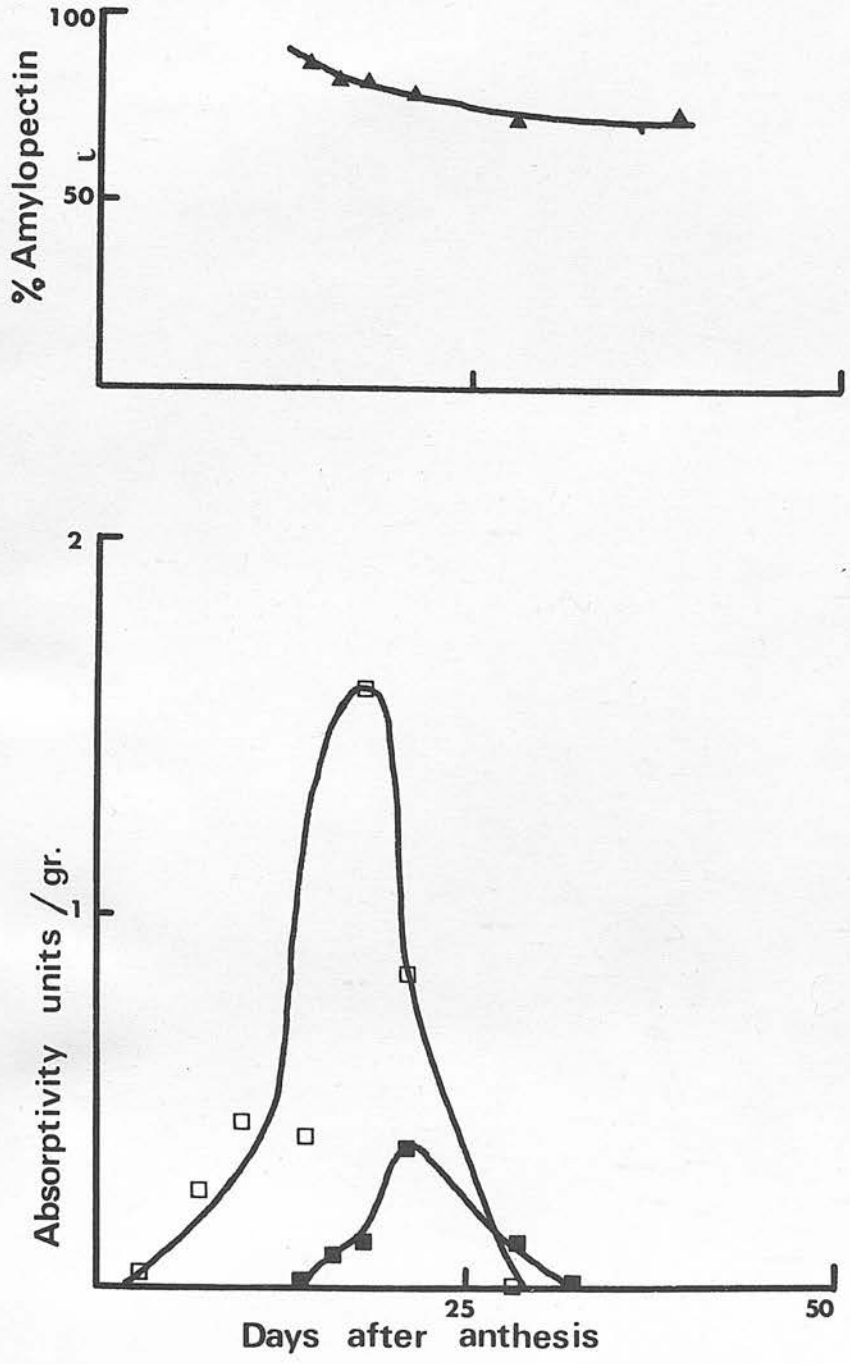
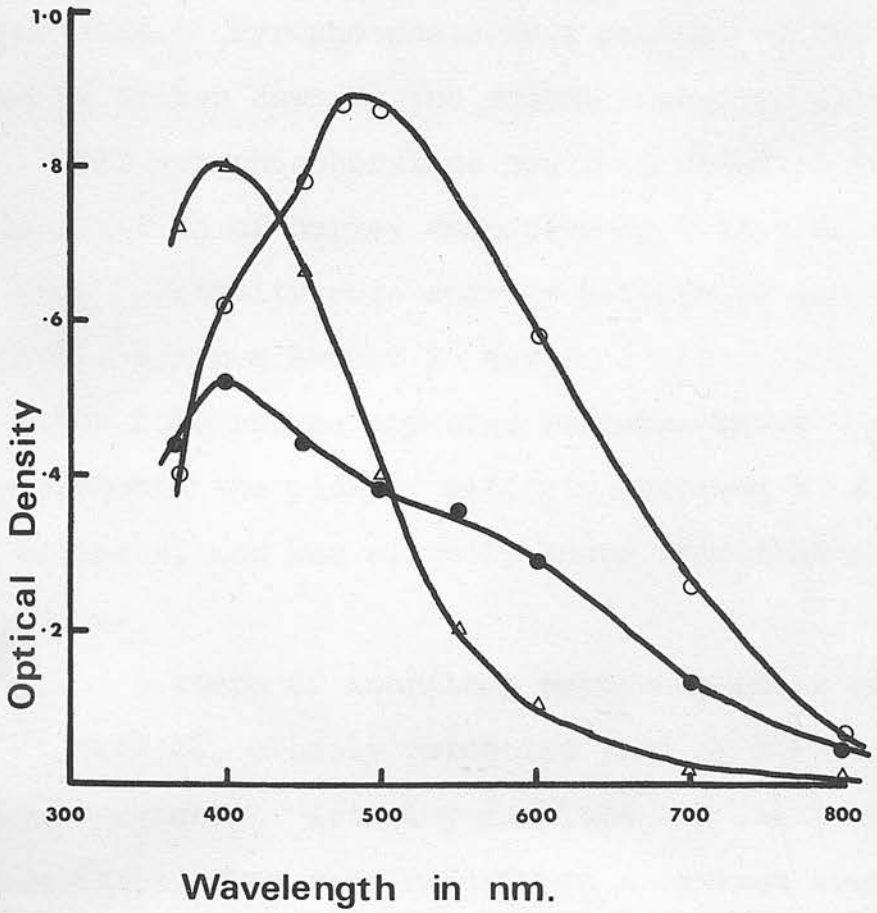

100
% Amylopectin
50
2
Absorptivity units / gr.
1
25
50
Days after anthesis

Figure 14. Absorption Spectra of amylopectin-iodide complex after incubation with soluble endosperm extract.

o pure amylopectin

Δ pure glycogen

● amylopectin after incubation with soluble endosperm extract.


1·0
·8
·6
·4
·2
Optical Density
300
400
500
600
700
800
Wavelength in nm.

UDPG and ADPG Pyrophosphorylase and Inorganic Pyrophosphatase

$$\underset{(ATP)}{UTP} + GIP \rightleftharpoons \underset{(ADPG)}{UDPG} + PP_i$$

$$PP_i \longrightarrow 2\ P_i$$

UDPG and ADPG may be synthesised from UTP or ATP respectively and GIP by the action of UDPG (ADPG) pyrophosphorylase. Pyrophosphate is a product of the reaction, and can be broken down by the enzyme inorganic pyrophosphatase.

UDPG pyrophosphorylase could be detected in the soluble fraction of barley endosperm by 7 days after anthesis (Fig. 15). Activity rose sharply between 12 and 15 days, attaining a maximum around 26 days.

The ADPG enzyme appeared somewhat later - around 12 days - although the peak of activity occurred at 22 days after anthesis, and was slightly lower than that of the UDPG enzyme.

The pattern of inorganic pyrophosphatase activity, shown in Fig. 16, closely resembled that of ADPG pyrophosphorylase. Activity remained low until 16 days after anthesis, then rose rapidly to a maximum around 23 days, thereafter declining abruptly. Slight activity could also be detected in the amyloplast fraction, from 12 days after anthesis.

Figure 15. UDPG and ADPG pyrophosphorylase activity in soluble endosperm extracts.

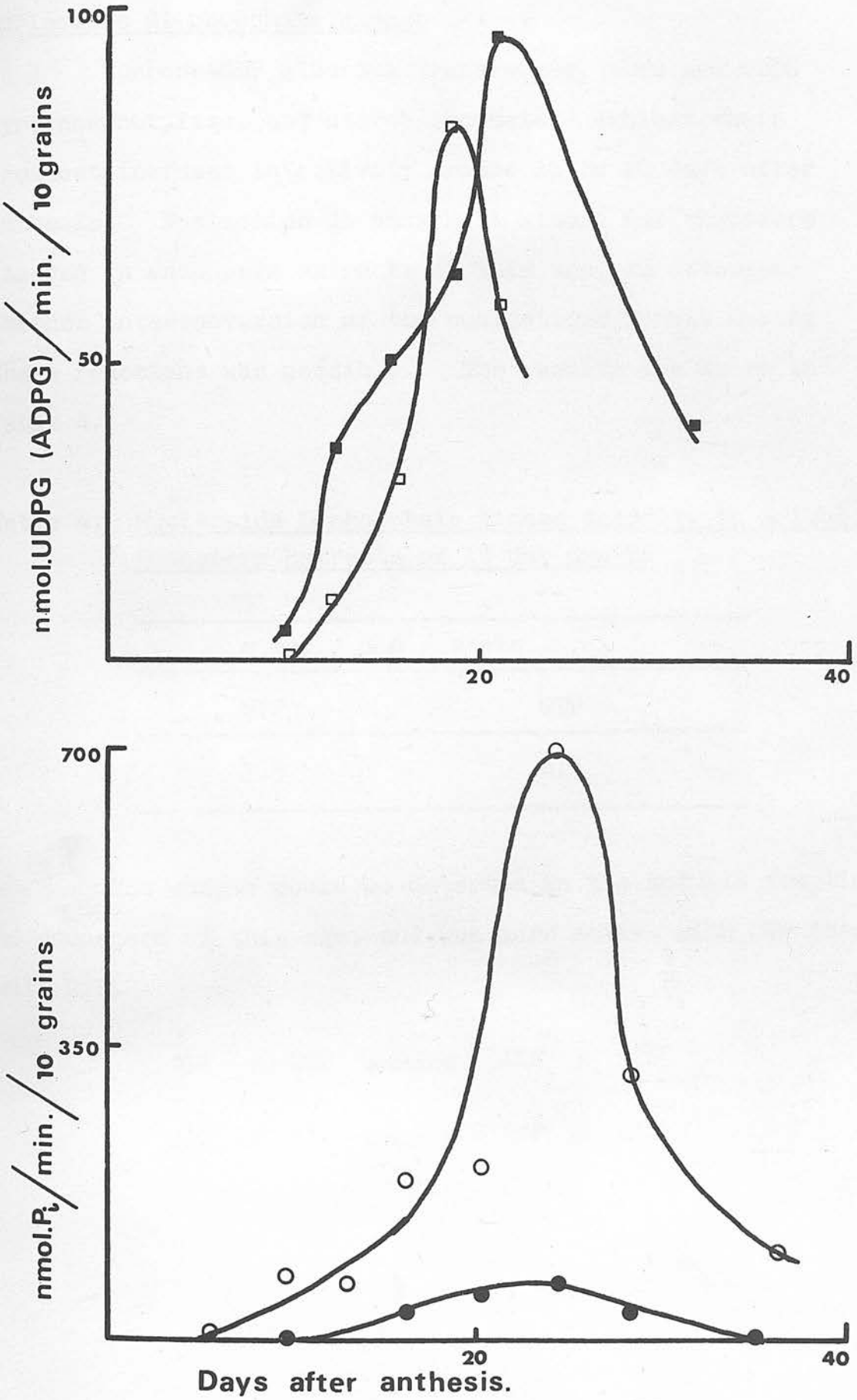
UDPG ■

ADPG. □

Figure 16. Inorganic pyrophosphatase in endosperm fractions.

soluble endosperm extract ○

amyloplast fraction. ●


100
50
n.mol.UDPG (ADPG) / min. / 10 grains
20
40
700
350
nmol.Pi / min. / 10 grains
20
40
Days after anthesis.

Nucleoside di-phosphate kinase

Sucrose-UDP glucosyl transferase, ADPG and UDPG pyrophosphorylase, and starch synthetase exhibit their greatest increase in activity around 15 to 20 days after anthesis. Nucleoside di-phosphate kinase was therefore assayed in endosperm extracts of this age, to determine whether interconversion of the nucleotides formed during these reactions was possible. The results are shown in Table 4.

Table 4. Nucleoside Diphosphate Kinase Activity in Soluble Endosperm Extracts of 15 day Grain.

n mol / min / grain	
UTP	GTP
7.5	4.5

The enzyme could be detected in the soluble fraction of endosperm of this age, and was more active with UTP than with GTP.

$$ADP + UTP \rightleftharpoons ATP + UDP$$

$$ADP + GTP \rightleftharpoons ATP + GDP$$

Hexokinase, Phosphoglucomutase and Glucose-6-phosphate keto isomerase

Hexokinase could be detected in both soluble and amyloplast fractions at 7 days after anthesis (Fig. 17) and increased rapidly in both cases during the 14 to 18 day period, reaching a steady maximum after 20 days. Activity in the amyloplast fraction was approximately half that of the soluble fraction.

$$\underset{(F)}{G} + ATP \longrightarrow \underset{(F6P)}{G6P} + ADP$$

Similar levels of activity were obtained with fructose.

$$F6P \rightleftharpoons G6P$$

$$G6P \rightleftharpoons GIP$$

Both glucose-6-phosphate keto isomerase and phosphoglucomutase were detected in soluble endosperm extracts and exhibited similar patterns of activity, shown in Figs. 18 and 19. These enzymes were present very early in development - 2 to 3 days after anthesis. There was no appreciable change in activity until 18 days, then a rapid increase between 20 and 22 days. Glucose-6-phosphate keto isomerase activity began to level off around 25 days, when phosphoglucomutase was declining slightly.

Figure 17. Hexokinase activity in endosperm fractions.

o soluble endosperm extract

● amyloplast fraction.

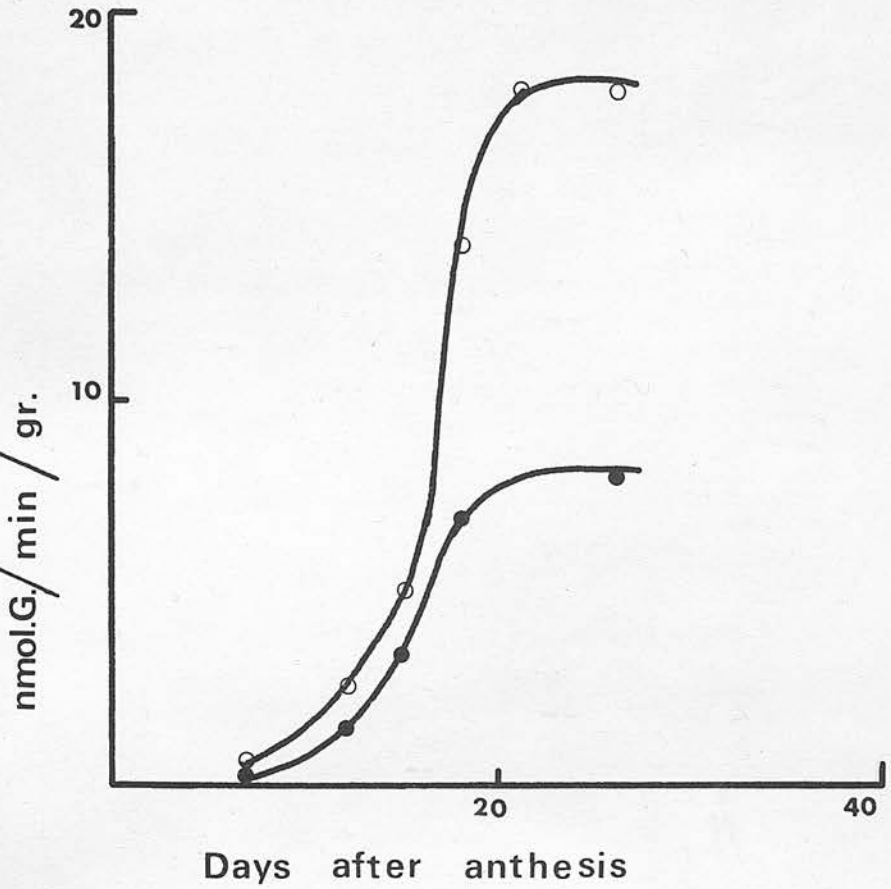
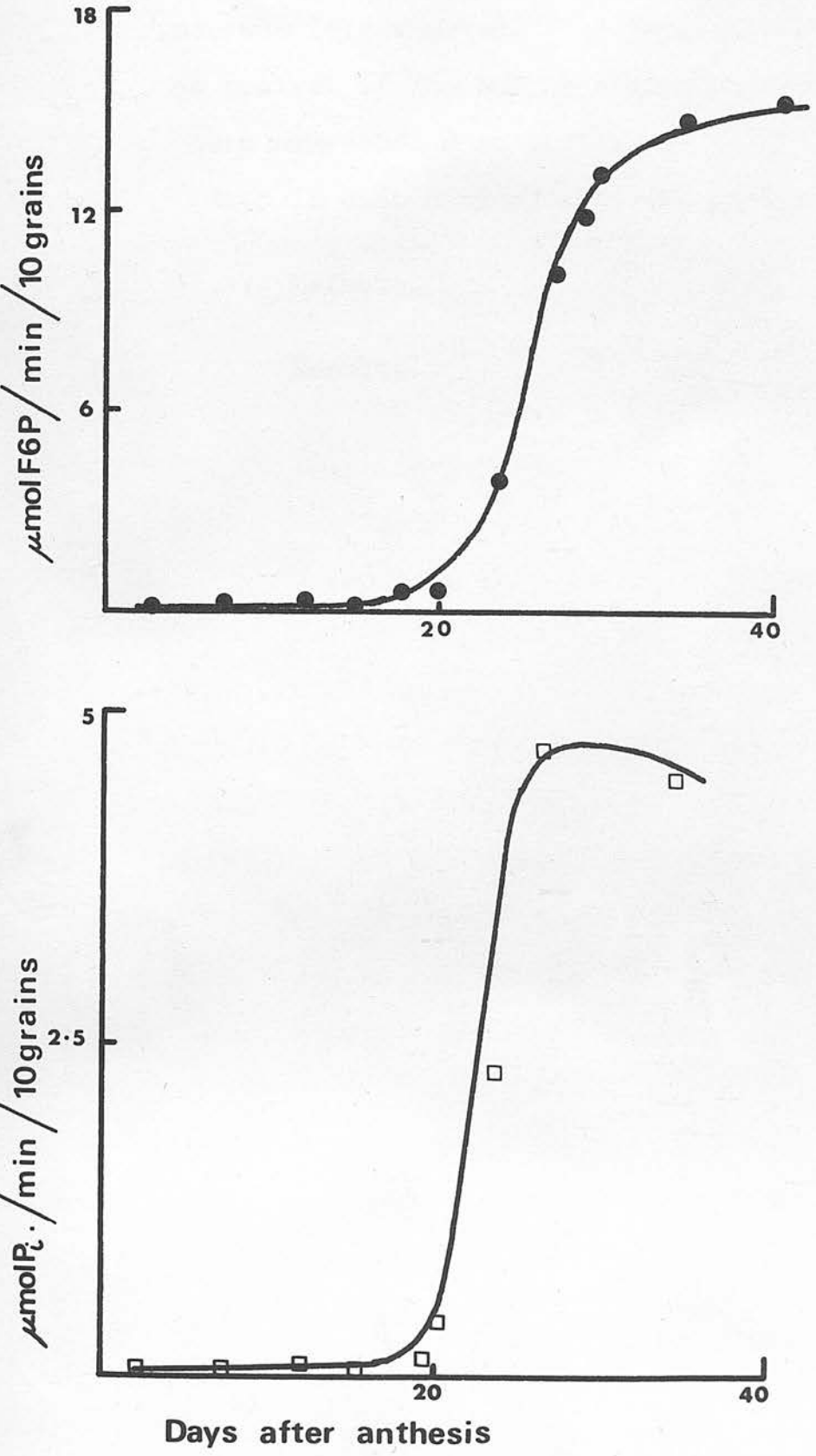

20
10
nmol.G./min/gr.
20
40
Days after anthesis

Figure 18. Glucose-6-phosphate keto isomerase activity in soluble endosperm extracts.

Figure 19. Phosphoglucomutase activity in soluble endosperm extracts.


18
12
6
μmol F6P / min / 10 grains
20
40
5
2·5
μmol Pi. / min / 10 grains
20
40
Days after anthesis

3. EXPERIMENTAL SECTION. PART 2.

The mechanism of starch biosynthesis in the absence of primer was investigated. In this context, the activity and control of the enzyme starch phosphorylase in barley endosperm were studied in detail.

This section is also divided into two parts:

3.A. Methods.

3.B. Results.

3.A. METHODS

3.A.1. Assay of Starch Phosphorylase. (α-1,4-glucan; orthophosphate glucosyl transferase. E.C.2.4.1.1.)

Enzyme activity was measured by the release of inorganic phosphate from GIP after incubation with endosperm extract for 2 hours at 25°C.[61] Soluble endosperm extracts were prepared as described in 2.A.2. (page 12). Aleurone extracts were obtained by scraping off the aleurone layer from the 25 day endosperm; this was homogenised and centrifuged in the same way as the endosperm extract.

Incubation mixtures contained:-

15 μmoles Tris-Maleate buffer, pH 6.2

10 μmoles GIP (dipotassium salt, Sigma Chemical Co.)

0.2 ml. endosperm or aleurone extract

in a total volume of 1 ml. When primed activity was measured, 0.3 ml of a 2.5% (w/v) solution of soluble starch (British Drug Houses) or corn endosperm amylopectin (Sigma Chemical Co.) was also included. After incubation the reaction was stopped with 0.5 ml of a 5% (w/v) solution of trichloroacetic acid (TCA), then 4 ml of 0.1N sodium acetate and 2 ml of acetate buffer, pH 4.0, were immediately added to raise the pH to 4.0, thus preventing any hydrolysis of unreacted GIP. The increase in inorganic phosphate was estimated using 1% ammonium molybdate and 2% ascorbic acid.[62] Values were adjusted for the non-enzymic breakdown of GIP using a blank from which endosperm extract had been omitted. Endogenous phosphate in the extract was corrected for by controls without GIP. Samples were also

tested for any increase in free glucose after incubation.[50] Results were expressed as μmoles P_i/grain. Total activity detected in the presence of added primer is referred to as 'primed activity', and activity with no added primer as 'unprimed'. 'Primed' activity was not calculated by subtracting unprimed activity from total values, since this presumed that, in the presence of primer, both primed and unprimed activity were concurrent. Unprimed activity was also expressed as a percentage of total activity.

The effect of inorganic phosphate on enzyme activity was estimated by including 0.1 ml of sodium di-hydrogen orthophosphate in the reaction mixture. The amount was varied to give a final concentration of 0.002 to 0.20 μM inorganic phosphate. The results given are an average of three experiments.

The variation of activity with pH was measured using Tris-Maleate buffers (0.05 M) at four different pH values, ranging from pH 5.2 to 8.2. (The ionic concentration of the buffer was not held constant.) Endosperm extracts were prepared in the appropriate buffer. The results are an average of three experiments.

The effect of adenine nucleotides on barley endosperm phosphorylase was also investigated. 3'5' cyclic adenosine monophosphate (cyclic AMP), ATP or AMP was included in the reaction mixture at a final concentration of 10^{-4}M or 10^{-2}M. Release of phosphate from these nucleotides by phosphatase activity was estimated by controls containing the appropriate nucleotide and endosperm extract, without GIP. In some cases the crude endosperm homogenate was also used as enzyme source.

3.A.2. Inorganic and Labile phosphate content of soluble endosperm extracts.

Inorganic phosphate in the soluble endosperm extract was determined by the Lowry-López method.[62] Total phosphate was measured after boiling the extracts with TCA for 10 minutes. The difference between total and inorganic phosphate was a measure of labile phosphate concentration.

3.A.3. Polyacrylamide gel electrophoresis

A Shandon disc electrophoresis apparatus, fitted with a Vokam power pack, was used. Electrophoresis was carried out on 7% polyacrylamide gels according to Maurer,[63] using gel system number 1, except that a 0.4% solution of oyster glycogen or corn endosperm amylopectin (Sigma Chemical Co.) was polymerised with some gels.[64] Soluble extracts from 80 endosperms of 7 day grain, 40 endospersm of 12-13 or 14-15 day grain, or 20 endosperms of 22 day grain, were prepared in 1 ml of buffer (0.1 M Tris-glycine pH 8.3) containing 10 mg. of sucrose. The same Tris-glycine without sucrose was used as reservoir buffer. A 0.05 ml sample of endosperm extract, together with bromophenol blue marker, was applied to each gel, and the current passed for about two hours at 5 mamp/tube, so that the marker just reached the end of the gel. They were then extracted by syringing with water and incubated overnight at 20°C in one of the following solutions:[64]

1. 3 ml 0.1M Citrate buffer, pH 5.0 + 3 ml 0.025 M GIP.
2. 3 ml 0.1M Citrate buffer, pH 5.0 + 3 ml 0.1M Phosphate buffer, pH 6.0.
3. 6 ml 0.1M Citrate buffer, pH 5.0.

4. 3 ml 0.5% glycogen or amylopectin in citrate buffer, pH 6.0 + 3 ml 0.025M GIP.

After incubation the gels were rinsed and stained with a solution of 0.01M I_2 + 0.014M KI, which coloured glycogen-containing gels light brown and amylopectin-containing gels blue. Zones with phosphorylase activity would be expected to stain blue-black after incubation in GIP. Both phosphorylase and amylase bands should appear colourless after incubation in citrate and phosphate, due to degradation of the glycogen or amylopectin. Only amylase degradation bands should be visible after incubation in citrate alone.

Rf values (that is, the distance moved by the component relative to the distance moved by the solvent front) were calculated, using bromophenol blue as an indication of the position of the solvent front.

3.A.4. Incorporation of Glucose from G*IP and UDPG* into starch

By using either GIP or UDPG radioactively labelled in the glucose moiety, it is possible to trace the incorporation of glucose from each glucosyl donor into starch in the absence of added primer.[38]

D-glucose-C_{14}(U)-1-phosphate, potassium salt (specific activity 277 mCi/m mol)(designated G*IP) and UDPG* (as described in page 15) were used. Both chemicals were obtained from the Radiochemical Centre, Amersham.

Reaction mixtures contained:-

0.2 ml endosperm extract

10-15 μmoles Tris-Maleate buffer, pH 6.2

G*IP or UDPG*

carrier GIP and/or carrier UDPG, as specified,

in a total volume of 0.5 ml.

Samples were incubated for periods of up to 24 hours at 37°C. Solid starch was then added as a carrier, and the insoluble material collected, washed, and counted for radioactivity as previously described (page 15).

Two sets of experiments were conducted:-

a) G*IP as radioactive source. 1.0 or 0.5 μCi were added per assay, together with 0.5 μmoles of carrier GIP and 0.6 μmoles of unlabelled UDPG where indicated. In some assays carrier GIP was omitted; this ensured that there were no contaminating carbohydrates in the reactants, since the G*IP was chromatographically pure.

The rate of incorporation of glucose with time was followed by setting up four identical pairs of incubations, one of each pair containing UDPG. Each pair was then incubated for a different length of time, and the amount of ^{14}C-glucose incorporated into the insoluble fraction determined as before. This experiment was repeated twice, using different incubation times.

Results were expressed as cpm/grain, or as percentage incorporation of glucose.

b) UDPG* as radioactive source. These incubations contained 0.10 or 0.05 μCi UDPG*, and 0.3 μmoles carrier UDPG and 0.1 μmole unlabelled GIP as indicated.

3.A.5. Autoradiography and β Amylolysis

The products formed in the radioactive assay system described in 3.A.4. were separated by paper chromatography. After the incubation a 0.05 ml sample was taken, and applied to Whatman No. 1 chromatography paper (46 cm x 57 cm) using a spotting tube. Soluble components were separated by descending chromatography for 20 hours using a solvent system of:- Propanol:Ethyl acetate:Water (6:1:3 v/v).[65] The chromatogram was dried at 80°C and labelled components visualised by exposure to an X-ray film (Kodak 'Kodirex', Estar base) for 1 week. The film was developed for 10 minutes with Kodak D-19 developer, washed with 1% acetic acid, and fixed for 30 minutes using Kodak 'Unifix'. Unlabelled sugars were detected by spraying the chromatogram with ammoniacal silver nitrate or anisidine-HCl reagent.[65] Reference solutions of GIP, UDPG, maltose, maltotriose, maltodecaose and maltotetraose were used in identifying reaction products. The maltotetraose and maltodecaose were the kind gift of Dr. Roger Stark (The Heriot Watt University, Edinburgh).

In some cases a 0.2 ml sample of the reaction mixture after incubation was treated with 0.1 ml of β amylase (1 mg/1 ml) (prepared from barley malt by Sigma Chemical Co.) for 1½ hours at 23°C, and a 0.075 ml sample applied to the chromatography paper. After exposure to the X-ray film, portions of the chromatogram corresponding to maltose and to the origin were cut out and the radioactive material eluted.[38] The appropriate area was cut into 0.2 cm strips and boiled with 1 ml of water for 5 minutes. This liquid was pipetted off and the strips

washed twice with 0.5 ml boiling water. Any remaining liquid was extracted from the paper by supporting the strips at the mouth of a centrifuge tube with Sellotape, and then centrifuging for 10 minutes at 12000 x g. The combined extracts and washings were evaporated to dryness, and any residue resuspended in 0.5 ml water by boiling, then counted for radioactivity as previously described (page 15).

3.A.6. Effects of amylolytic attack on phosphorylase activity

In these experiments soluble endosperm extracts were preincubated with glucoamylase (α-1,4-glucan glucohydrolase. E.C.3.2.1.3.) or β amylase (α-1,4-glucan maltohydrolase. E.C.3.2.1.2.) before being assayed for phosphorylase activity.

50 mg of glucoamylase (specific activity 2700 mg glucose/minute/gm at 55°C pH 4.5, prepared from Rhiszopus mould by Sigma Chemical Co.) was dissolved in 1 ml of acetate buffer, pH 4.0, and 2 mg β amylase (specific activity, 15 mg maltose/minute/mg at 20°C, pH 4.8) in 2 ml of the same buffer. The soluble endosperm extract was prepared in 8 ml of Tris-Maleate buffer, 0.05M, pH 6.2. Two incubations were set up, each containing 2 ml of extract and 0.2 ml glucoamylase or β amylase respectively. Control incubations contained extract and glucoamylase or β amylase which had been boiled for 1 hour. The mixtures were incubated for 2 hours at 30°C, then 1 ml was withdrawn from each tube and dialysed for 1 hour at 4°C against 500 ml of 0.01M Tris-Maleate buffer, pH 6.2. The remaining 1 ml of each incubation was kept at 4°C without dialysis during this period. Dialysed and undialysed

solutions were then assayed for phosphorylase activity, either by release of inorganic phosphate or by the incorporation of glucose from G*IP as previously described in 3.A.1 and 3.A.4.

Reference solutions of β amylase and glucoamylase with a solution of soluble starch, showed that 0.2 ml glucoamylase released 3 mg glucose,[50] and 0.2 ml β amylase released 6 mg maltose[66] per 10 mg starch after incubation for 2 hours at 30°C pH 4.0.

3.A.7. Partial purification of phosphorylase by DEAE-cellulose column chromatography

Soluble endosperm extracts from freshly gathered field barley (7 days, 14 days and 22 days after anthesis) were prepared as previously described in 2.A.2. (page 12), except that the buffer used was 0.01M Tris-Maleate, pH 7.0. An equal volume of saturated ammonium sulphate was added to the extract very slowly, at 0°C, and the mixture stirred for a further 20 minutes. The precipitated fraction (P) was collected by centrifugation for 20 minutes at 34,000 x g in an 8 x 50 ml Angle head (MSE High speed 18), dissolved in 2 ml of buffer and dialysed for 2 hours against the same buffer. The supernatant solution (S) was also dialysed. Samples of the original amyloplast and soluble endosperm fractions, together with (P) and (S) were assayed for protein content[47] and primed phosphorylase activity.[61,62]

1 ml of (P) was applied to a DEAE-cellulose column (1 cm x 20 cm) previously equilibrated with the Tris-maleate

buffer.[37] The sample was eluted in a total volume of 110 ml by a linear discontinuous sodium chloride (NaCl) concentration gradient, from 0 to 1M in Tris-Maleate buffer, pH 7.0.[37] 10 ml of buffer were first applied, then 20 ml each of NaCl solutions of 0.10M; 0.25M; 0.50M; 0.75M; and 1.00M. 5 ml fractions were collected and dialysed overnight against the same buffer.

Each fraction was assayed for primed phosphorylase activity by phosphate release from GIP[61,62] (see 3.A.1). Fractions showing activity were also assayed for glucose incorporation from G*IP either in an unprimed system or with added maltose as a low molecular weight primer,[37] (see 3.A.4).

The protein[47] and carbohydrate[46] content of each fraction were also determined.

3.B. RESULTS

3.B.1. Starch Phosphorylase

$$GIP + [G]_n \rightleftharpoons [G]_{n+1} + P_i$$

Starch phosphorylase could be detected in soluble endosperm extracts from as early as 3 days after anthesis (Fig. 20). Using soluble starch as a primer, activity increased rapidly to a maximum around 22 days, declining immediately to low values which were subsequently maintained during the 30 to 40 day period. Somewhat higher activities, following the same developmental pattern, could be obtained using a corn endosperm amylopectin primer, but soluble starch was used routinely because it could be dissolved more readily than the amylopectin.

Most of the activity was located in the starchy endosperm rather than in the aleurone layer (see Table 5).

Table 5. Primed Phosphorylase Activity in Aleurone and Endosperm Fractions of 25 day Grain

Results are averages of 2 expts.

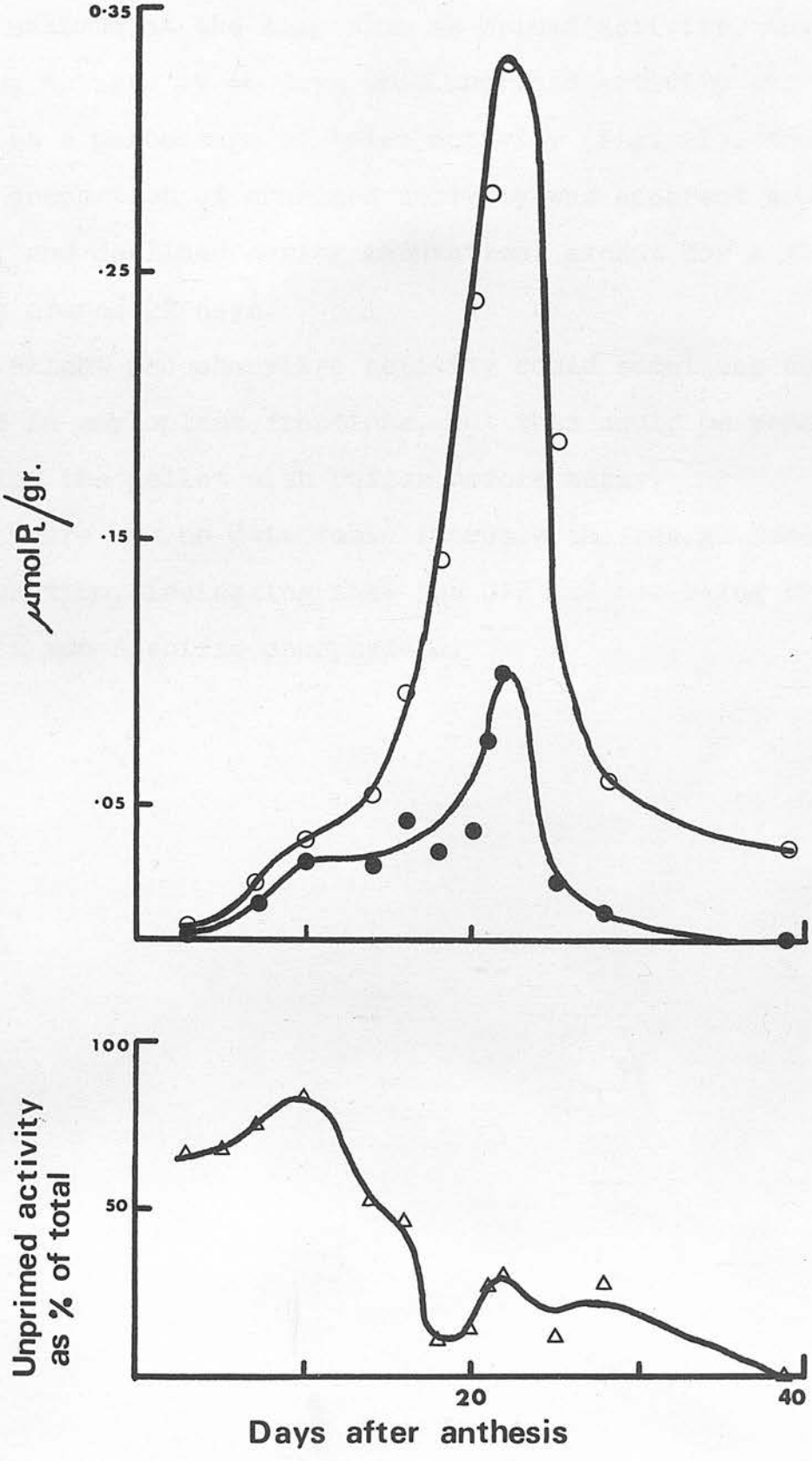
Fraction	μmoles P_i/grain
Starchy endosperm (corrected for aleurone)	0.135
Aleurone	0.015

A similar pattern of activity was observed in the absence of any added primer. Unprimed activity was only slightly lower than primed during the first 10 days after

Figure 20. Phosphorylase activity in soluble endosperm extracts.

- o total activity in presence of soluble starch primer
- ● unprimed activity.

Figure 21. Unprimed phosphorylase expressed as a % of total phosphorylase activity in soluble endosperm extracts in presence of starch primer.


0·35
·25
·15
·05
μmol P_i/gr.
100
50
Unprimed activity as % of total
20
40
Days after anthesis

anthesis, but increased more slowly from 10 to 20 days, to reach a maximum at the same time as primed activity, then declining to zero by 40 days. When unprimed activity was expressed as a percentage of total activity (Fig. 21), the highest proportion of unprimed activity was apparent around 10 days, and declined during maturation, except for a slight increase around 22 days.

Slight phosphorylase activity could sometimes be detected in amyloplast fractions, but this could be removed by washing the pellet with buffer before assay.

There was no detectable increase in free glucose during the incubation, indicating that the GIP was not being broken down by a non-specific phosphatase.

3.B.2. Inorganic and Labile phosphate content of soluble endosperm extracts

It can be seen from Fig. 22 that levels of inorganic phosphate remained low until 11 days after anthesis, rising rapidly to a peak at 25 days. Labile phosphate could not be detected before 10 days, but increased sharply during the 11 to 16 day period, reaching a maximum around 30 days. The ratio of labile to inorganic phosphate was highest at 14 days, subsequently declining abruptly, although a small increase was apparent around 29 days.

3.B.3. Effect of Inorganic phosphate on phosphorylase

Addition of inorganic phosphate to the incubation medium inhibited phosphorylase activity measured in the direction to starch synthesis. Table 6 indicates that:-

(a) primed and unprimed activity were inhibited to differing extents,

(b) the inhibition was age dependent.

Primed activity in 7 and 14 day extracts was inhibited by increasing phosphate concentrations to a greater extent than in 22 day extracts. Conversely, unprimed activity was inhibited to a lesser degree in younger grain than in more mature ones.

When this unprimed activity was expressed as a percentage of total activity (Fig. 23) it became evident that the extent of inhibition of unprimed activity at each concentration of phosphate was lower in young grains than in older ones.

Figure 22. Inorganic and labile phosphate content of soluble endosperm extracts.

- ● Inorganic phosphate
- ○ Labile phosphate
- ▲ Labile/inorganic phosphate.

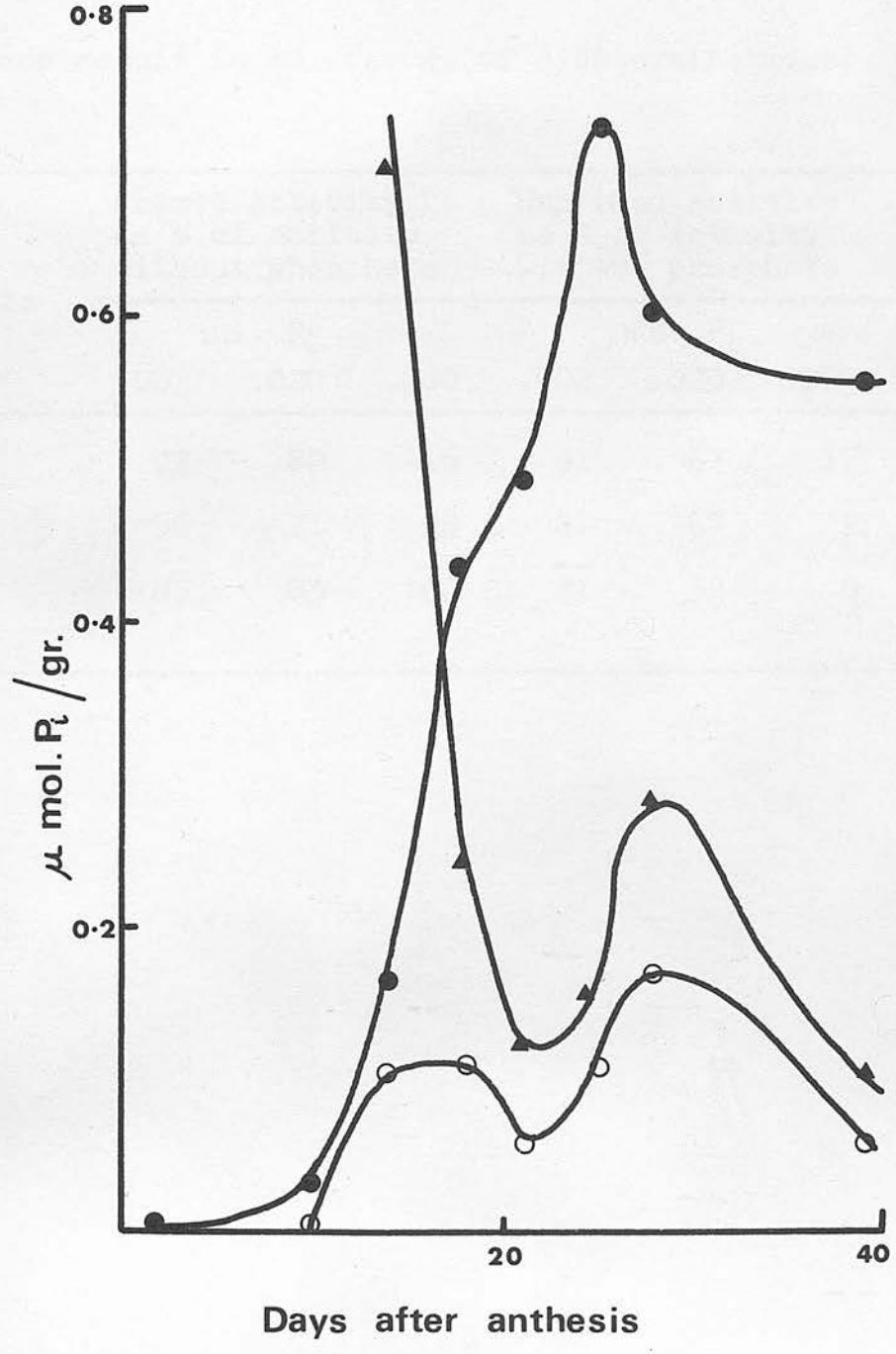

0·8
0·6
0·4
0·2
μ mol. Pt /gr.
20
40
Days after anthesis

TABLE 6. Inhibition of Phosphorylase in Soluble Endosperm Extracts by Inorganic Phosphate

(Each result is an average of 3 determinations)

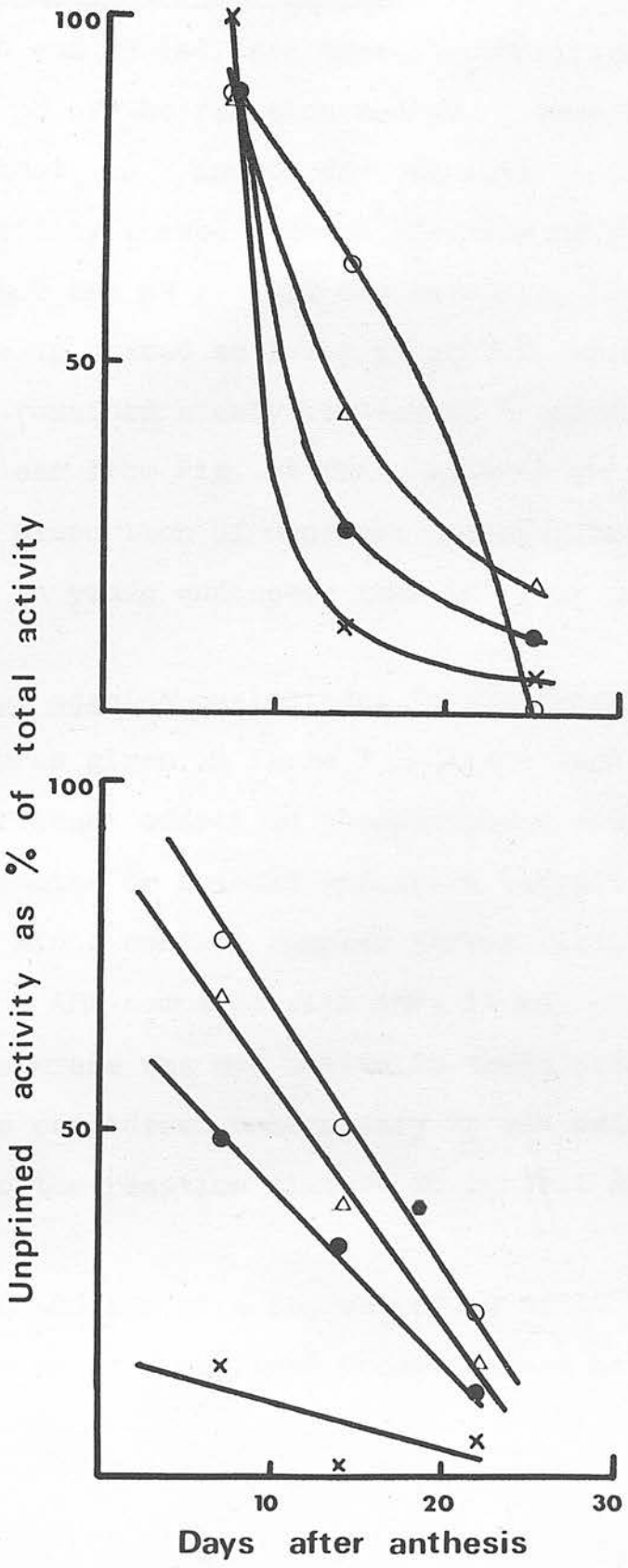
Age in days after anthesis	Primed Activity as % of Activity without phosphate			Unprimed Activity as % of Activity without phosphate		
	μM. P_i			μM. P_i		
	.002	.020	.200	.002	.020	.200
7	92	80	16	91	67	17
14	96	71	28	81	67	2
22	85	83	64	71	52	0

Figure 24. pH dependence of phosphorylase activity in soluble endosperm extracts. Unprimed activity as a % of total activity.

- o pH 5.2
- Δ pH 6.2
- ● pH 7.2
- x pH 8.2

Figure 23. Effect of inorganic phosphate on phosphorylase activity - unprimed as a % of total activity.

- o no added phosphate
- Δ .002 μM P
- ● .020 μM P
- x .200 μM P


100
50
100
50
Unprimed activity as % of total activity
10
20
30
Days after anthesis

3.B.4. pH dependence of phosphorylase

Figs. 24 and 25 indicate that phosphorylase activity varies with the pH of the reaction medium. From Fig. 25 it can be seen that, in 7 and 14 day extracts, both primed and unprimed activity showed a broad spectrum of pH dependence between pH 6 and pH 7. 22 day extracts, however, showed a sharp increase in primed activity at pH 7.2, whereas unprimed activity remained steady between pH 6 and pH 7.

It is clear from Fig. 24 that, as with phosphate inhibition, the proportion of unprimed activity is less dependent on pH in young endosperm than in older grain.

3.B.5. Effect of adenine nucleotides on phosphorylase

The figures given in Table 7 indicate that cyclic AMP had no significant effect on phosphorylase activity in endosperm homogenates or soluble endosperm extracts of 7 and 14 day grain. Since control samples showed little release of P_i from cyclic AMP compared with AMP, it was presumed that 3'5' cyclic diesterase was not active in these extracts, and it was therefore considered unnecessary to add aminophyllin (theophyllin) to the reaction mixture to inhibit cyclic AMP breakdown.

Both ATP and AMP at a concentration of 10^{-4}M, were inhibitory to primed and unprimed phosphorylase activity.

Figure 25. pH dependence of phosphorylase activity.

(1) 7 day soluble endosperm extracts.

(2) 14 day soluble endosperm extracts.

(3) 22 day soluble endosperm extracts.

In each case:-

o total activity with added primer.

● unprimed activity.

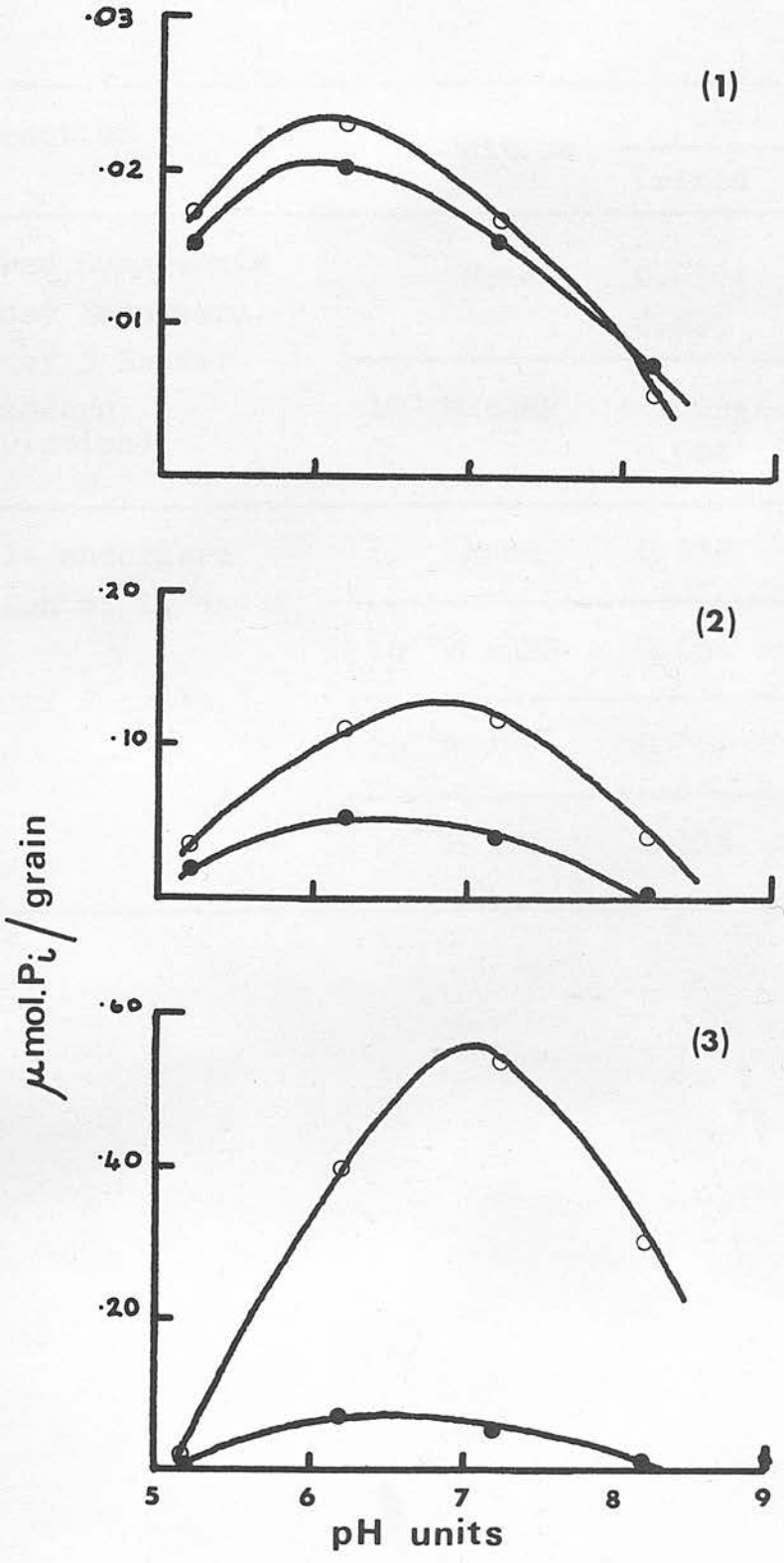

·03
·02
·01
(1)
·20
·10
(2)
·60
·40
·20
(3)
μmol.Pi / grain
5
6
7
8
9
pH units

TABLE 7. Effect of Adenine Nucleotides on Barley Endosperm Phosphorylase

Fraction	Addition	µmol P_i/Grain	
		Primed	Unprimed
Filtered Homogenate of 7 day Endosperm. (Mean of 3 Expts. ± Standard deviation)	None	0.056± 0.025	0.032± 0.003
	10^{-2}M cAMP	0.069± 0.004	0.024± 0.002
Soluble endosperm fraction of 14 day grain. (Mean of 2 Expts.)	None	0.038	0.019
	10^{-4}M cAMP	0.034	0.012
	10^{-4}M AMP	0.018	0.013
	10^{-4}M ATP	0.015	0.005

3.B.6. Separation of Phosphorylase isoenzymes by polyacrylamide gel electrophoresis

Soluble extracts of barley endosperm could be fractionated into two zones of phosphorylase activity by electrophoresis on polyacrylamide gels containing glycogen (Fig. 26). Extracts from 7 and 12-13 day grain (A1 and B1) showed two dark bands after incubation with GIP; a slow moving component (1), Rf 0.01, and a faster moving component (2), Rf 0.13. In 7 day extracts component (1) was more active, while (2) predominated in 12-13 day extracts. Plate 2 shows bands obtained with 7 and 14-15 day extracts. Here the current was passed for 3 hours to render the slow moving component (1) more easily visible.

With 22 day extracts (Fig. 26. C1) component (1) could no longer be detected, and two bands were observed at Rf 0.11 and Rf 0.13, corresponding to component (2).

Substitution of orthophosphate for GIP in the reaction mixture resulted in colourless bands of the same Rf values as the dark bands, (A2, B2, C2). Colourless amylase bands were also apparent, at Rf 0.028 and 0.42; these remained unchanged throughout the 7 to 22 day period. They could be distinguished from the colourless bands due to degradative phosphorylase activity by omitting orthophosphate from the incubation mixture, in which case only amylase bands were visible, (A3, B3, C3).

When glycogen was added to the incubation mixture instead of being polymerised in the gel, a similar pattern of activity was observed, except that the bands were fainter, possibly due to the restricted diffusion of such a large

Figure 26. Electrophoresis of endosperm extracts on polyacrylamide gels.

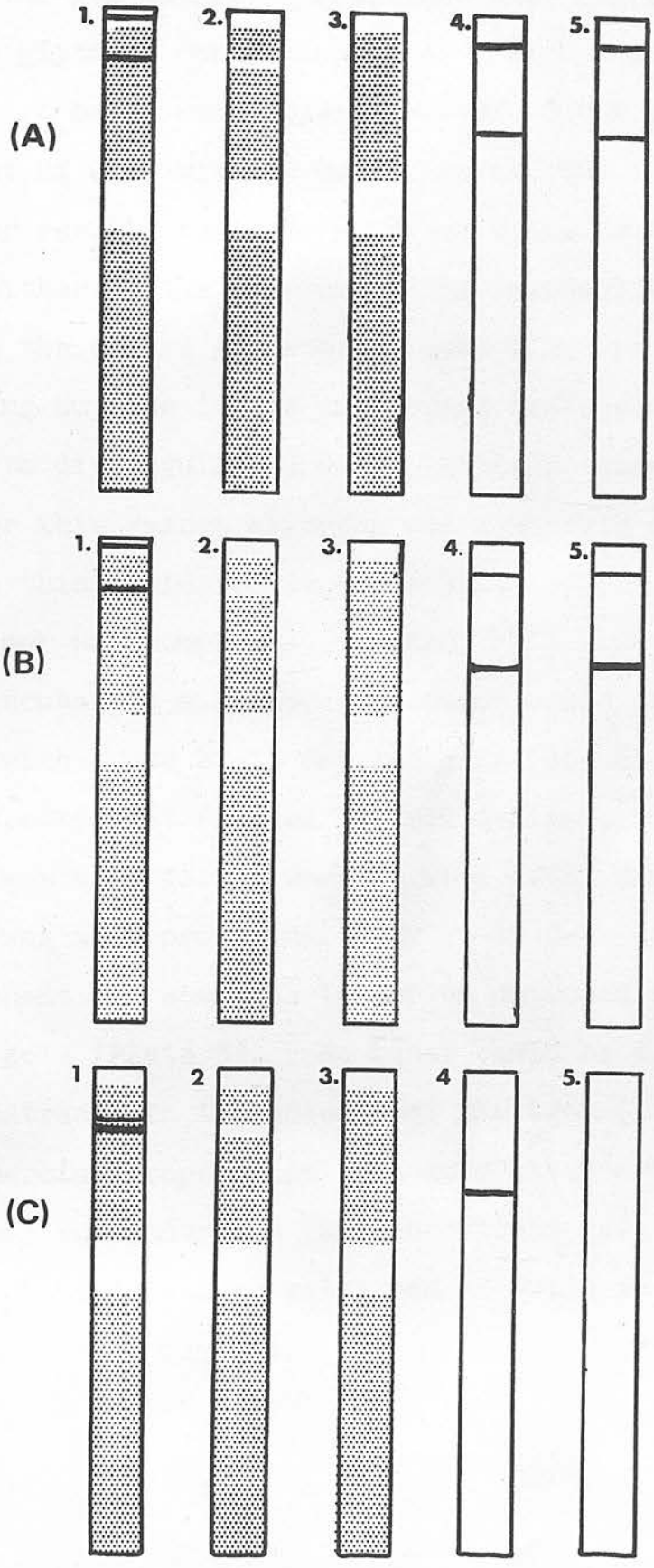
(A) 7 day soluble endosperm extracts

(B) 12-13 day soluble endosperm extracts

(C) 22 day soluble endosperm extracts.

1. Gels incubated in GIP and citrate buffer.
2. Gels incubated in phosphate and citrate.
3. Gels incubated in citrate.
4. Gels incubated in glycogen in citrate and GIP.
5. Gels incubated in GIP and citrate.

Indicates glycogen gel. 


(A)
1.
2.
3.
4.
5.
(B)
1.
2.
3.
4.
5.
(C)
1
2
3.
4
5.

molecule into the gel matrix. Fractions also migrated faster than in glycogen containing gels. With 7 and 12-13 day extracts dark bands were observed at Rf. 0.045 and 0.23 (A4, B4) and at Rf 0.24 with 22 day extracts (C4).

Similar results could be obtained using amylopectin as a primer, either in the gels or in the incubation mixtures. However, since the entire gel stained dark blue (possibly due to contaminating amylose in the commercial amylopectin) it was difficult to distinguish bands of synthetic phosphorylase activity. For this reason glycogen was routinely employed as a primer in this series of experiments.

If primer was completely omitted, both from the gels and from the incubation mixture, dark bands could still be distinguished with 7 and 12-13 day extracts (A5, B5). With 7 day extracts component (1) was clearly defined, but component (2) was very faint, whereas with 12-13 day extracts component (2) was more prominent. By 14-15 days after anthesis component (1) could no longer be detected in glycogen-free gels (Plate 3). No bands could be distinguished with 22 day extracts in the absence of glycogen (Fig. 26 C5).

A commercial preparation of muscle phosphorylase b (Boehringer Co.) run under the same conditions gave only one band, at Rf 0.066 in glycogen gels, and Rf 0.133 in glycogen free gels incubated in glycogen.

Plate 2. Electrophoresis of soluble endosperm extracts on acrylamide gels containing glycogen.

(a) 7 day endosperm

(b) 15 day endosperm

Plate 3. Electrophoresis of soluble endosperm extracts on glycogen-free acrylamide gels.

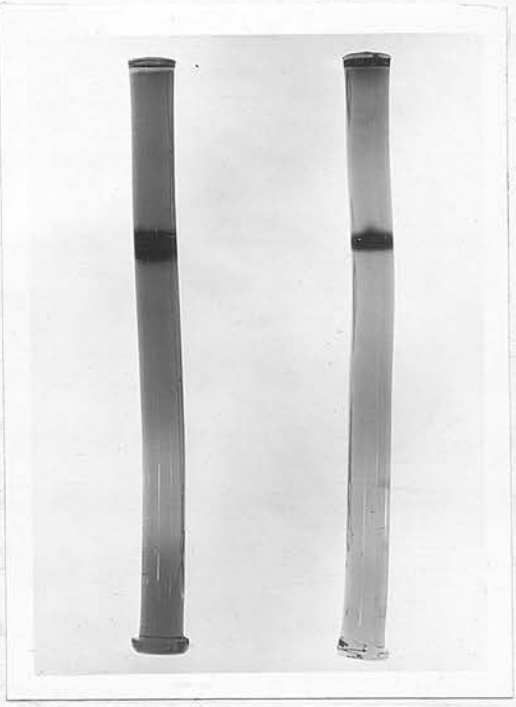
(a) 7 day endosperm

(b) 15 day endosperm

All gels were incubated in GIP and citrate buffer overnight, then stained with KI-I_2 reagent as described in the Methods section.

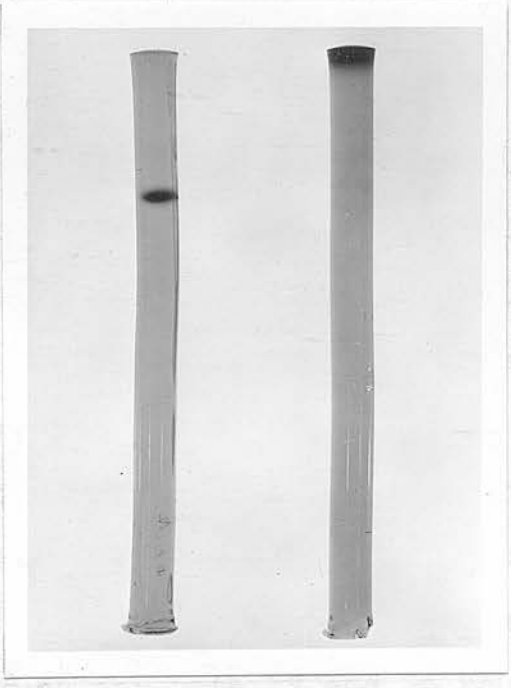
b. a.

2.



b. a.

3



3.B.7. Incorporation of glucose from GIP and UDPG into starch in the absence of added primer

The results of glucose incorporation experiments are expressed in Table 8. From 8(a) it can be seen that in the presence of UDPG approximately 2% of the G*IP was incorporated into an insoluble fraction over a period of 24 hours, by soluble endosperm extracts of 3 to 5 day grain. This figure remained unchanged when UDPG was added after the initial 12 hours of incubation rather than at the beginning. Most of the activity was localised in the soluble fraction.

Incorporation of glucose could also be detected when total incubation time was reduced to 12 hours (Table 8(b)). Soluble extracts from 2-3 day endosperm could only incorporate glucose from G*IP in the presence of UDPG. In 5 day extracts however, UDPG appeared to exert an inhibitory influence.

Soluble extracts of 16 day endosperm showed considerable incorporation of glucose into insoluble material, but this was apparently unaffected by UDPG (Table 8(c)).

The rate of incorporation of glucose from G*IP by 5 day endosperm extracts is depicted in Fig. 27. It is clear that addition of UDPG caused a slight activation during the initial 10 hours, but a strong inhibitory effect appeared as the length of incubation was prolonged.

Table 9 shows the incorporation of glucose from UDPG* into starch by starch synthetase activity. The extent of incorporation was found to be reduced when GIP was included in the reaction mixture for both 12 and 24 hour incubations with 5-7 day soluble endosperm extracts.

TABLE 8. Incorporation of ^{14}C-Glucose from G*IP into starch in Absence of Added Primer

(a) 24 Hour incubation 1.0μ Ci (3.3 n moles) G*IP and 0.6 μ moles UDPG/assay.

Fraction of 3-5 day endosperm	Treatment	% GIP incorporated	No. of Expts.
Soluble	UDPG added after 12 hours	1.7	2
	UDPG added at beginning of incubation	1.9	5
Amyloplast	UDPG added after 12 hours	0.2	2

(b) Effect of UDPG. 12 Hour incubation. 1.0 μ Ci G*IP per assay (Soluble endosperm extracts)

Age (Days)	cpm/Grain	
	+ UDPG (0.6 μ moles)	- UDPG
2-3	74	0
3-4	754	11
5	49	185

(c) 16 Day soluble Endosperm Extract. 0.5 μ Ci G*IP + 0.1 μMole carrier GIP 24 hour incubation
(Values are mean of two determinations)

cpm/grain	
UDPG (0.6 μ moles)	- UDPG
3,072	3,239

Figure 27. Effect of UDPG on rate of incorporation of ^{14}C-glucose from G*IP into starch

+ UDPG ○

no UDPG ●

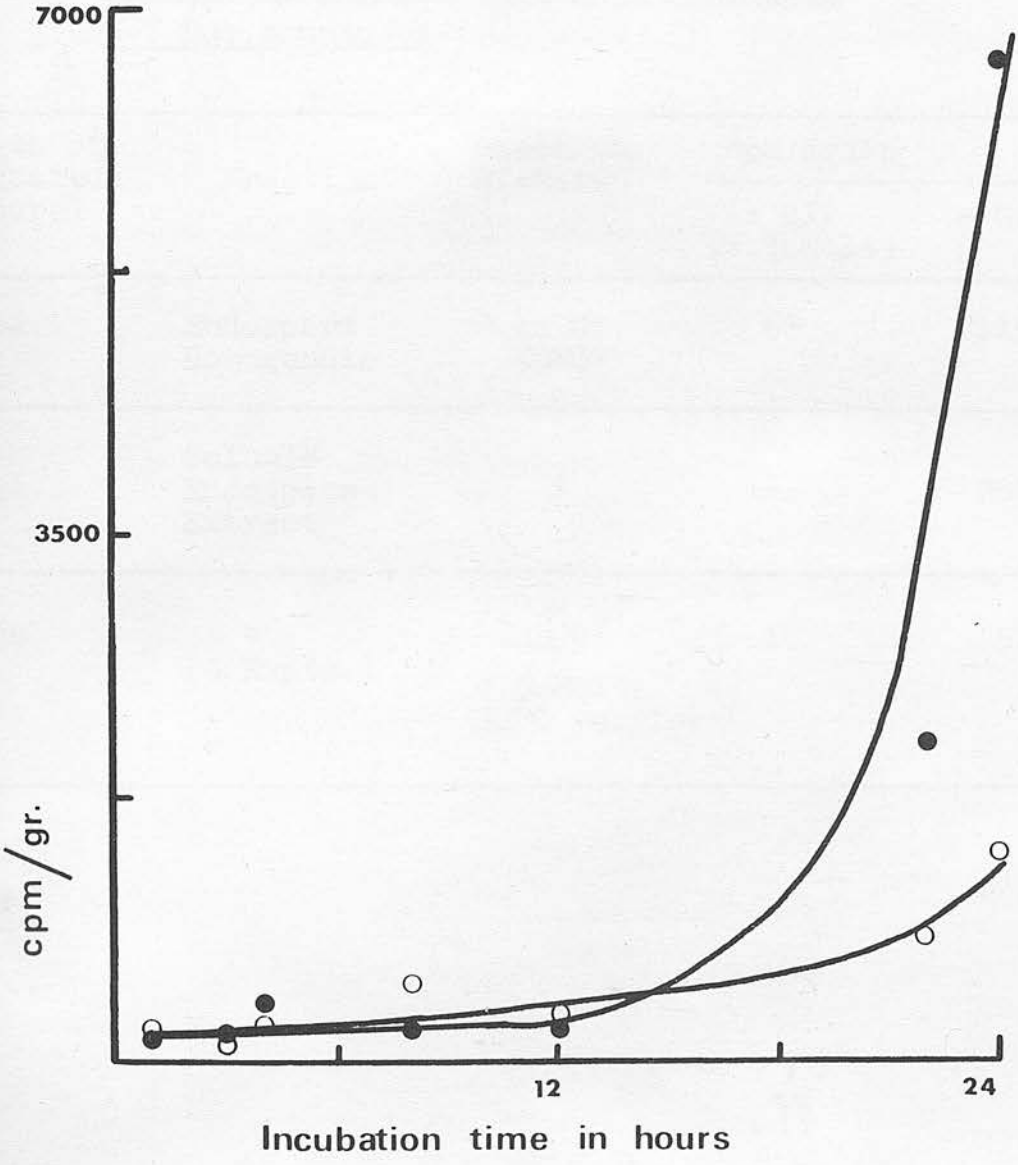

7000
3500
cpm/gr.
12
24
Incubation time in hours

TABLE 9. Incorporation of ^{14}C-glucose from UDPG* into Starch in absence of added primer. 5-7 Day endosperm.

Length of incubation (hours)	Fraction	Reaction Mixture	cpm/grain	
			+ GIP (0.1µMole)	- GIP
12	Endosperm Homogenate	0.1µ Ci UDPG*	66	119
24	Soluble Endosperm Extract	"	41	268
24	" (3 Expts.)	0.05µ Ci UDPG* 0.3µMoles UDPG carrier	18	51

However no incorporation from UDPG* could be detected by 2-3 day grains in incubation of 4, 12 or 24 hours.

In some cases barley ears 5-7 days after anthesis were allowed to stand in water overnight before endosperm extracts were prepared. It was then observed that an increased incorporation of glucose into starch was obtained when both UDPG and GIP were added (Table 10).

TABLE 10. Effect of Standing Overnight on Incorporation of ^{14}C-glucose into starch by soluble fraction of 5-7 day endosperm

Reaction Mixture	Treatment of ears	cpm/grain	
		+ UDPG (0.6 μ Moles)	- UDPG
0.5μ Ci G*IP + 0.1μ Mole Carrier GIP	Left in H_2O overnight before dissection. 24 hour Incubation	2276	779
	Dissected immediately. 24 hour Incubation	1408	2042
		+ GIP (0.1μ Mole)	- GIP
0.1μ Ci UDPG*	Left in H_2O 12 hour Incubation	184	82
	Dissected immediately 12 hour Incubation	41	268

3.B.8. Autoradiography and β Amylolysis

Chromatography of endosperm extracts incubated with G*IP or UDPG* showed the accumulation in the reaction mixture of a labelled material which remained immobile in the solvent system used, (Plates 4 and 5). A series of labelled substances lying between the origin and the maltose reference spot could also be observed; these were especially marked with incubations containing both G*IP and UDPG, or GIP + UDPG*. Since suitable labelled reference substances were not available, these components could not be positively identified. They could, however, be removed, and the proportion of immobile material reduced, by incubation of the extract with β amylase. A concomitant increase in labelled maltose was also observed, (Plates 4 and 5, (c) and (d)). It was therefore presumed that the slow-moving substances were maltosaccharides, containing 4 or more glucose units, and the immobile material was an α-1,4-glucan closely resembling amylose.

It can be seen from Table 11 that the polysaccharide synthesised from G*IP alone gave rise to a much higher proportion of maltose than material synthesised from UDPG*, even taking into account the lower levels of radioactivity added with UDPG*. Incubations containing G*IP + UDPG, or GIP + UDPG* produced similar quantities of maltose after β amylase treatment.

(Since maltose will be produced by amylolytic attack on both immobile material and maltosaccharides, it will therefore appear disproportionately large when compared to the amount of immobile material alone.)

Plate 4. Chromatogram showing incorporation of ^{14}C-glucose from G*IP into α-1,4-glucans.

a) Incubation containing G*IP + UDPG.

b) Incubation containing G*IP.

c) Incubation containing G*IP + UDPG, treated with β amylase.

d) Incubation containing G*IP, treated with β amylase.

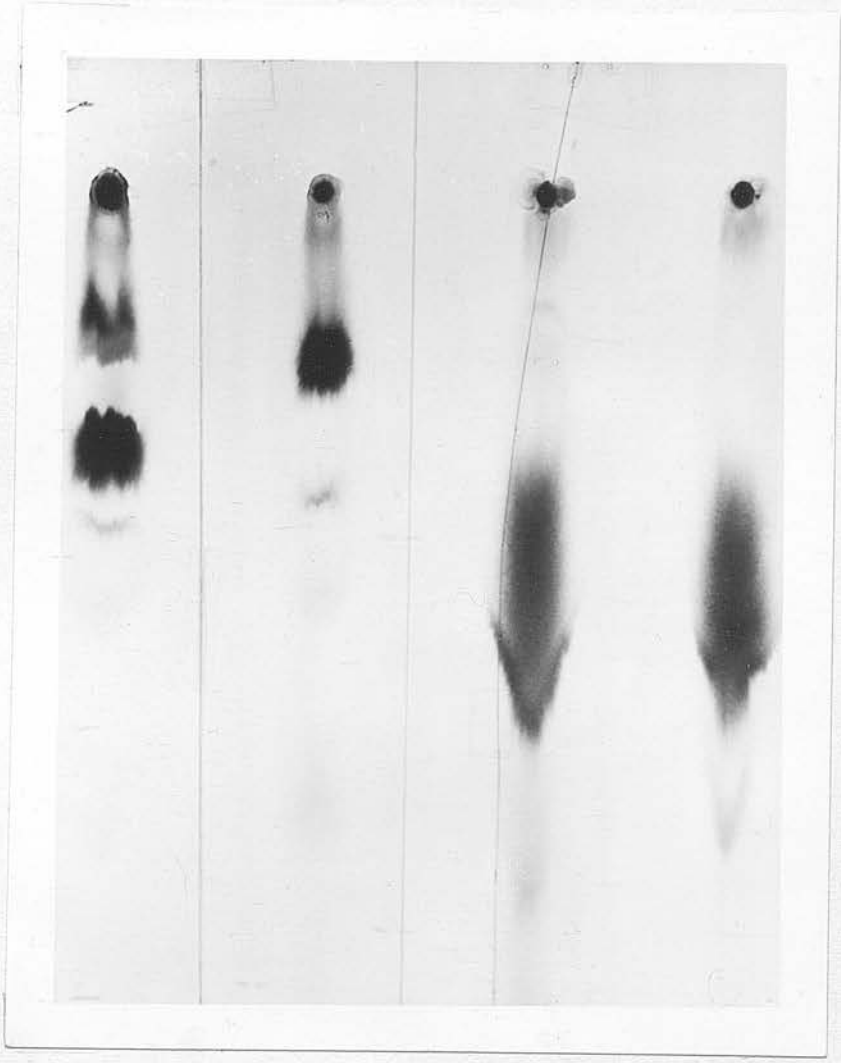

Origin
GIP
Maltotriose
Maltose
Glucose
a
b
c
d

Plate 5. Chromatogram showing incorporation of ^{14}C-glucose from UDPG* into α-1,4-glucans.

a) Incubation containing UDPG* + GIP.

b) Incubation containing UDPG*.

c) Incubation containing UDPG* + GIP, treated with β amylase.

d) Incubation containing UDPG*, treated with β amylase.

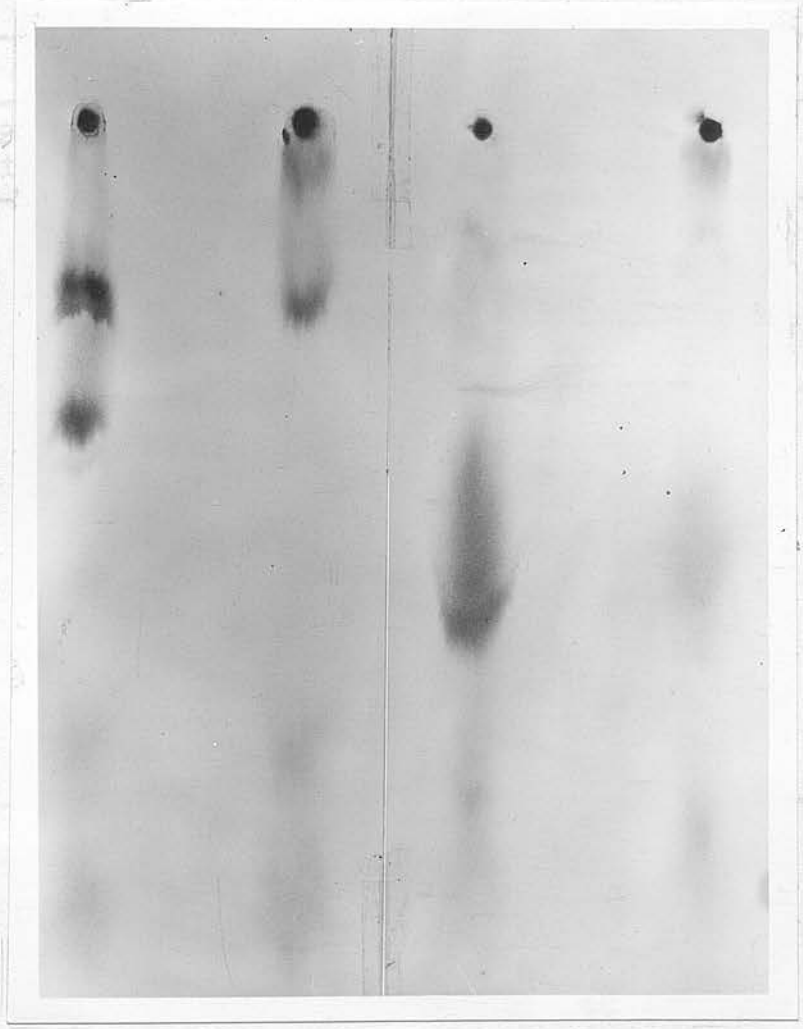

Origin
UDPG
Maltotriose
Maltose
Glucose
a
b
c
d

TABLE 11. Radioactivity of spots eluted from Chromatograms, showing the effect of β amylase attack.

Incubation Mixture	β Amylase treatment	cpm	
		At origin	At Maltose
G*IP + UDPG	Before	3440	498
	After	1747	8313
GIP + UDPG*	Before	635	482
	After	194	1597
G*IP	Before	2411	384
	After	1810	11858
UDPG*	Before	1281	388
	After	1043	646

3.B.9. Effect of amylolytic attack on phosphorylase activity

Carbohydrate components could be detected in the soluble endosperm extract at very early stages and may supply endogenous primers for phosphorylase. Pre-incubation of the extract with amylolytic enzymes would therefore be expected to have some effect on 'unprimed' phosphorylase activity in these extracts.

The results obtained from a series of incubations with β amylase or glucoamylase are given in Table 12. Both primed and unprimed activity in soluble extracts from 7 and 15 day endosperm increased considerably after incubation with glucoamylase. The extent of this increase was reduced by dialysis, but was still significant. No significant change in activity was found after incubation with β amylase.

Effects of incubation with glucoamylase were also investigated using that fraction of the soluble endosperm extract obtained by precipitation with 50% saturated ammonium sulphate as the source of phosphorylase, (Table 13). As with the crude extract, glucoamylase treatment caused a considerable increase in both primed and unprimed phosphorylase. No unprimed activity was detectable in control fractions pre-incubated with boiled glucoamylase. This may mean that ammonium sulphate precipitation destroys unprimed activity except after glucoamylase treatment. However, in later experiments unprimed activity could be detected in similar ammonium sulphate fractions. It seems probable that partial purification with ammonium sulphate may render the enzyme more unstable, so that it is more likely to be destroyed during a two hour

incubation with boiled glucoamylase.

The incorporation of glucose from G*IP into starch was also found to be activated by pre-incubation with glucoamylase, (Table 14). The increase in activity was more pronounced when UDPG was omitted from the reaction mixture.

TABLE 12. Effect of Glucoamylase and β amylase on Phosphorylase Activity in Soluble Endosperm Extracts.

(Results are means of 3 expts. ± standard deviation).

(a) 7 Day Endosperm

Treatment	Primed Activity µmol.P /1000 grain		Unprimed Activity µmol.P /1000 grain	
	Not Dialysed	After Dialysis	Not Dialysed	After Dialysis
+ Glucoamylase	57 ± 3	28 ± 3	50 ± 3	28 ± 8
Boiled Glucoamylase	30 ± 7	10 ± 1	15 ± 1	6 ± 2
+ β Amylase	15 ± 0.5	9 ± 2	10 ± 3	5 ± 4
Boiled β Amylase	15 ± 1	9 ± 3	14 ± 3	1 ± 1

(b) 15 Day Endosperm

Treatment	Primed Activity µmol.P /1000 grains		Unprimed Activity µmol.P /1000 grains	
	Not Dialysed	After Dialysis	Not Dialysed	After Dialysis
+ Glucoamylase	80 ± 10	45 ± 9	75 ± 3	33 ± 2
Boiled Glucoamylase	15 ± 4	15 ± 5	16 ± 4	9 ± 3
+ β amylase	21 ± 4	15 ± 2	18 ± 5	14 ± 5
Boiled β amylase	19 ± 2	21 ± 7	14 ± 4	6 ± 3

TABLE 13. Effect of Glucoamylase on Phosphorylase Activity in Endosperm Extracts Purified by Ammonium Sulphate Precipitation
(7 day endosperm)

Fraction	Treatment	µmoles P_i/mg Protein	
		Primed	Unprimed
Soluble endosperm extract	Untreated	1.31	0.62
50% saturated NH_4SO_4 precipitate	+ Glucoamylase		
	Dialysed	4.72	4.44
	Not dialysed	7.94	6.83
	+ Boiled glucoamylase		
	Dialysed	0.55	0
	Not dialysed	0.88	0

TABLE 14. Effect of Glucoamylase on Incorporation of Glucose from G*IP into Starch in Absence of Primer.

Treatment	Age in days after anthesis	Substrate	cpm/grain	
			+0.6µmol. UDPG	No UDPG
+ Glucoamylase	3	0.25µ Ci G*IP	333	367
Boiled Glucoamylase		0.5µmol. GIP	130	
+ Glucoamylase	5	0.25µ Ci G*IP	863	3650
Untreated		0.5µmol. GIP	257	293
+ Glucoamylase	5	0.5µ Ci G*IP	2583	6359
Untreated		0.1µmol. GIP	319	432

3.B.10. Preliminary purification of phosphorylase by DEAE-cellulose chromatography

Table 15 shows phosphorylase activity of extracts from 7, 12 and 22 day endosperm during purification. Some activity was associated with the amyloplast fraction at each age investigated; this was probably due to contamination by soluble enzyme, since these amyloplast fractions were not washed before assaying, (see page 59).

Much of the soluble enzyme in 7 and 12 day extracts could be concentrated by precipitation with 50% saturated ammonium sulphate; with 22 day extracts a lower proportion of the enzyme could be precipitated by this procedure.

Fig. 28(1) indicates that extracts from 7 day endosperm contained two peaks of phosphorylase activity physically separable by DEAE-cellulose column chromatography. The first peak (fraction number 4) is designated Phosphorylase I and the second peak (fraction 16) Phosphorylase II. Only Phosphorylase I was capable of transferring glucose from G*IP into starch in the absence of added primer, although Phosphorylase II showed slight activity with a maltose primer.

Extracts of 12 day endosperm gave three peaks of phosphorylase activity (Fig. 28(2)), two of which were very close together (fractions 3, 5 and 6) and probably corresponded to Phosphorylase I of 7 day extracts. The third peak (fraction 15) corresponded to Phosphorylase II. Incorporation of glucose from G*IP into starch in the absence of added primer was negligible in all fractions except fraction 15, where some activity could be detected. Addition of maltose

increased the extent of glucose incorporation by all fractions.

Only one fraction of phosphorylase activity was separable from 22 day extracts. This was eluted in fractions 9 and 10, and did not appear to correspond to either phosphorylase I or II of 7 and 12 day extracts. This enzyme was almost inactive in the absence of starch, and was only slightly activated by the addition of maltose.

No carbohydrate could be detected in any of the fractions eluted from the column.

TABLE 15. Phosphorylase Activity during Purification

Fraction	Primed Phosphorylase Activity					
	μmol.P_i/mg Protein			Total Activity of Fraction in μmol.P_i		
	7 Day	12 Day	22 Day	7 Day	12 Day	22 Day
Amyloplast	1.73	1.47	1.33	4.32	1.23	19.0
Soluble Endosperm Extract	2.40	1.73	2.86	21.6	7.8	85.8
50% Sat. NH_4SO_4 Precipitate	3.68	2.32	1.81	11.1	3.7	20.4
50% Sat. NH_4SO_4 Supernatant solution	1.42	1.85	1.87	3.58	0.96	7.2
DEAE Cellulose fractions — Fraction No.						
3	-	4.6	-	-	1.5	-
4	9.4	-	-	2.1	-	-
5	-	9.6	-	-	2.2	-
6	-	6.1	-	-	1.4	-
9	-	-	0.8	-	-	0.3
10	-	-	6.3	-	-	2.4
15	-	2.5	-	-	0.5	-
16	9.3	-	-	1.9	-	-

Figure 28. Fractionation of phosphorylase by DEAE-cellulose chromatography.

(1) 7 day endosperm extracts.

(2) 14 day endosperm extracts.

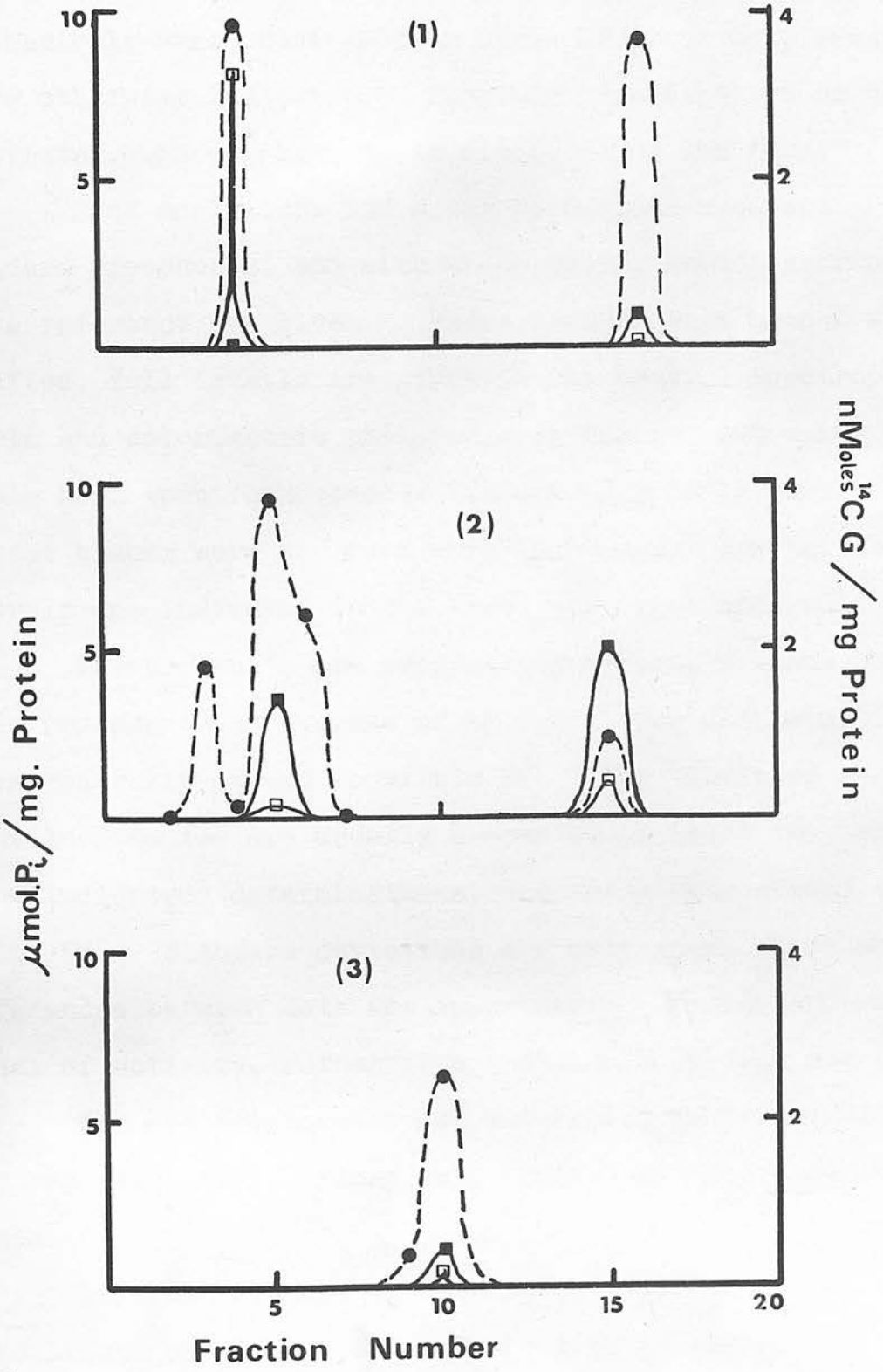
(3) 22 day endosperm extracts.

In each case:-

- - -●- - - phosphorylase activity measured in μmoles Pi/mg protein.

—■— incorporation of ^{14}C-glucose into starch from G*IP in presence of maltose primer. (measured as nmoles ^{14}C-glucose/mg protein).

—□— incorporation of ^{14}C-glucose in absence of added primer.


(1)
(2)
(3)
10
5
4
2
μmol.Pi/mg. Protein
nMoles 14C G / mg Protein
5
10
15
20
Fraction Number

Accuracy and Precision of Experimental Techniques and Results

'Analar' reagents and solvents were used throughout. Biochemicals were obtained from Sigma Chemical Co., except where otherwise indicated. Possible contamination of the substrates, where relevant, is discussed in the text.

Most analytical and assay techniques used are standard procedures, and with these only a brief description and a reference are given. Where methods have been at all modified, full details are given in the text. Spectrophotometric and colorimetric analyses were carried out using a double beam spectrophotometer (Unicam. S.P.800). Appropriate reagent blanks were included with each assay, and suitable controls (as indicated in the text) were also assayed.

Where results are presented graphically, each point given represents an average of at least four determinations, which generally agreed to within 5%. For tabulated results, the values quoted are usually a mean of at least two (or more where indicated) determinations, and these also agreed to within 5%. Standard deviations are only given where small differences between data are important. In many experiments, trends of activity, rather than individual values, are compared.

The electrophoretic and autoradiographic procedures were repeated several times, and typical experimental results presented.

With the glucose incorporation experiments the number of replicate samples which could be treated was severely limited by the availability of fresh barley in the very young

stages after anthesis, as well as by the experimental conditions which required the dissection of very large quantities of small endosperms, followed by long incubation periods. In some cases only one determination could be carried out under each set of conditions. However sufficient replicate samples were assayed wherever possible to establish key patterns of activity. Overall trends of activity were compared from all experiments involving incorporation of radioactive substrates. Emphasis was not placed on any individual values.

Points expressed graphically were averages of at least 4 determinations. The curves were drawn freehand without statistical fitting.

4. DISCUSSION

4.1. Major biochemical constituents

The pattern of changes in the major biochemical constituents of maturing barley grains closely resembled those recorded in wheat,[8] peas,[56] and barley,[12] and could be correlated with observed morphological changes. A rapid gain in water content, and consequently in fresh weight, around 10 to 14 days after anthesis marked the beginning of a gradual increase in soluble protein and enzyme activity. There was a close correspondence between total carbohydrate content and dry weight, both of which remained low until 15 to 16 days. The consequent divergence of fresh and dry weights between 10 and 14 days resulted in elongation and increased turgidity of the endosperm, with minimal gain in amyloplast size. The rapid accumulation of carbohydrates between 20 and 40 days after anthesis accounted for a corresponding rise in dry weight, together with an increase in amyloplast diameter.

During the initial 20 day period carbohydrates accumulated in the soluble fraction of the endosperm before being transferred to the insoluble fraction. Only a small proportion of the total soluble carbohydrates was due to reducing sugars, and much of the remainder can be attributed to sucrose, which has been established as the predominant sugar in the early stages of seed development in wheat[8,67] and peas.[68] The relative contribution of reducing sugars to the dry weight fell as the percentage content of starch increased. These results obtained with barley endosperm are consistent with a mechanism whereby soluble oligosaccharides

are synthesised at the expense of mono- and di-saccharides. These then provide a precursor pool which is initially depleted during the onset of rapid starch synthesis, but subsequently reforms from the continuing supply of sugars.

^{14}C assimilation experiments in wheat[69] have demonstrated that the CO_2 fixed during photosynthesis in the stems and flag leaf of the plant is mainly incorporated into sugars - sucrose, 3- and 4-unit oligosaccharides, and fructans. Part of this is translocated into the developing inflorescence during the period immediately prior to anthesis, but probably accounts for less than 10 percent of the final dry weight of the ear. Carr and Wardlaw[70] showed that photosynthesis in wheat leaves declined after anthesis, while that in the ear increased. They also demonstrated significant assimilation of ^{14}C by the awns (in awned varieties of wheat) as well as by the glumes, lemma, and palea during illumination. The testa-pericarp fraction of the barley endosperm has been shown to be capable of light dependent oxygen evolution. Carbon compounds may thus be provided by the ear itself, and possibly also by the outer structures of the grains, during part of the maturation period.

As development progressed, soluble protein became increasingly associated with the amyloplast fraction at the expense of the soluble endosperm extract. Much of this may be attributed to the synthesis of storage protein bodies which fractionated with the amyloplasts, but may also reflect some enzyme protein contained in, or attached to, the amyloplasts.

The final stages of maturation were marked by a gradual desiccation causing progressive yellowing of the stems and leaves, shrivelling of the grains, and a slight reduction in amyloplast size. The activity of most enzymes investigated began to decline just prior to the onset of desiccation. However La Berge et al.,[16] found that β amylase activity in wheat remained constant after 30 days, and Duffus and Rosie[17] observed an increase in latent β amylase in barley towards maturity (see also 14, 15). It seems probable that certain enzymes required for the initial utilization of reserve starch may be stored in a latent form in the mature grain, although α amylase is known to be synthesised de novo[9,10] during germination.

4.2. Sucrose Metabolism

The developmental pattern of the enzymes involved in sugar metabolism indicated that the period between 10 and 14 days after anthesis represented a crucial stage in enzyme activity. Most of the enzymes investigated could be detected prior to this period, but at very low levels of activity. Reaction rates began to increase around 14 days, reaching a maximum between 20 and 30 days, then declining. Thus the increase in enzyme activity immediately preceded gains in dry weight.

The high levels of sucrose-UDP glucosyl transferase (relative to most other enzymes detected) during the 10 to 14 day period indicate that this enzyme may be important for

the initial utilization of translocated sucrose. Pressey[71] found that at physiological pH's sucrose-UDP glucosyl transferase catalysed sucrose breakdown much faster than sucrose synthesis. In view of the low levels of invertase in barley endosperm it seems likely that most of the sucrose is converted to nucleotide sugars. A contrasting situation can be observed in maize, where Tsai and Nelson[53] observed a peak of invertase activity at 12 days after anthesis, before any detectable sucrose-UDP glucosyl transferase. Shannon[72,73] has suggested that translocated sucrose in the maize plant is cleaved by invertase activity during entry to the endosperm, and diffuses as monosaccharides through the endosperm cells. Sucrose would then be resynthesised in cells active in starch synthesis. There would appear to be no evidence for the existence of such a mechanism in barley.

In barley endosperm transfer of glucose to UDP by sucrose-UDP glucosyl transferase activity greatly exceeded that to ADP, confirming results obtained with maize endosperm,[32] tapioca tubers (Manihot utilissima)[33] and cotyledons of Vicia faba.[74] De Fekete and Cardini[32] also noted that the ADP reaction was inhibited by uridine nucleotides. It is probable, therefore, that little ADPG can be synthesised by this route, and thus UDPG is the major product.

4.3. Starch Synthesis

Starch synthetase associated with the amyloplasts of barley endosperm was found to utilise both UDPG and ADPG as glucosyl donors, although activity with ADPG was approximately double that with UDPG. On the other hand, the soluble enzyme appeared slightly more active with UDPG than with ADPG. This contrasts with other results where soluble synthetases have been observed to be more specific towards ADPG than starch granule bound synthetases of the same tissues.[75,76] Tanaka and Akazawa[75] showed that the soluble enzyme from spinach leaf chloroplasts was completely ADPG specific, whereas the bound enzyme from spinach seeds was active with both glucosyl donors. The bound enzyme from soya bean leaf (Glycine max.) however, appeared ADPG specific.[77] Frydman and Cardini[76] examined bound starch synthetase from several plant sources, and they concluded that, whereas in tissues accumulating reserve starch, the enzyme could utilize both UDPG and ADPG, only ADPG would serve as donor in Geranium leaf and soya bean. They also found that mechanical disruption of the starch granules caused a loss of activity towards UDPG, while increasing activity with ADPG. They therefore suggested that the soluble and bound synthetases were modifications of the same enzyme, the difference in substrate specificity being due to the formation of an insoluble amylose-enzyme complex (see also 75, 78). Variations in the structure of the polysaccharide component (i.e. whether amylose or amylopectin) could possibly further modify the properties of the particulate enzyme system.

The results of the present investigation would agree in outline with this hypothesis. Young barley grains contain mainly soluble synthetase and the proportion of amyloplast bound enzyme increases with the concentration of starch in the grain. Since the soluble enzyme can utilise both UDPG and ADPG, and since the bound enzyme is predominantly ADPG specific, complexing with starch would seem to increase the ability to utilise ADPG. This contrasts with Frydman and Cardini's[76] results where binding to starch granules increased ability to utilise UDPG. An initial predominance of UDPG-linked starch synthetase was detected in barley endosperm during the 4 to 10 day period. It is probable that the 'bound' enzyme prepared during this period reflects soluble enzyme attached to the outer membrane of the amyloplasts (which contain little starch at this stage). As more starch is synthesised it could remain attached to the enzyme giving rise to bound starch synthetase (i.e. an amylose-enzyme complex), with a heightened affinity for ADPG. Thus the exact nature of the 'bound' starch synthetase preparation will alter during development as the starch content of the amyloplast increases.

Alternatively it is also possible that the bound and soluble starch synthetases are isoenzymes with different substrate requirements. Multiple forms of starch synthetase have been isolated from waxy and non waxy strains of rice[79,80] and maize.[81] However, these were both prepared from soluble starch synthetases and were both specific for ADPG, although different primer requirements were observed. Thus although it is possible that the soluble enzyme may exist as different

isoenzymes, it remains probable that the bound enzyme is due to complexing of the soluble enzyme with newly synthesised starch.

(The different primer requirements of these isoenzymes will be discussed in more detail later, in relation to phosphorylase isoenzymes.)

It has also been proposed that the formation of this polysaccharide-starch synthetase complex is a controlling factor in the regulation of amylose/amylopectin ratios.[82,83,84] Although the peak of branching enzyme activity in barley endosperm occurred relatively early in development, before the period of most rapid starch synthesis, the amylopectin content declined from 14 to 22 days, when branching enzyme activity was at a maximum. No debranching activity, (measured by the release of glucose from starch limit dextrin[85]) could be detected, although such activity may have been obscured in crude extracts by glycolysis. It was observed that strains of rice[82] and maize[83] differing in amylose content contained similar levels of branching enzyme activity. Clearly the extent of branching is not governed by branching-enzyme alone. It was noted, however, that waxy rice grains contained very much lower levels of bound starch synthetase than non waxy grains.[82] Similar results were obtained by Murata and Akazawa.[84] It was suggested that the formation of a complex between newly synthesised amylose and starch synthetase reduces access to branching enzyme, and consequently lowers the proportion of amylopectin formed. In the absence of a bound synthetase, all amylose is open to attack from branching enzyme, resulting in a highly branched 'waxy' or 'glutinous'

starch. In non-waxy starches, such as that of the present study, initial preponderance of soluble synthetase allows the formation of a high proportion of amylopectin, which declines as the extent of bound synthetase increases.

The progressive association of branching enzyme itself with the amyloplasts must also be noted in this context. It seems plausible that this soluble enzyme becomes trapped and inactivated as the amyloplast grows by apposition of starch. Thus mechanical disruption of the amyloplasts increases detectable activity.

Variation in amylose/amylopectin ratios may also be explained by preferential transfer from a particular glucose donor into starch. Murata et al.,[86] showed that bound starch synthetase preparations from developing rice grains transferred glucose residues from ADPG to amylopectin, whereas those from UDPG appeared to be transferred equally to amylopectin and amylose. Nelson and Rines[87] have also reported the apparent absence of UDPG-starch synthetase from amyloplast preparations of waxy maize, although the enzyme could be detected in amyloplasts of non-waxy grains. However, since activity with ADPG was not tested, this may merely reflect the lack of bound starch synthetase in waxy grains described above. Although differential incorporation into amylose and amylopectin was not investigated, no evidence for such a mechanism could be detected in the present work. UDPG-starch synthetase appeared predominant during the period of highest amylopectin content, and the appearance of the ADPG enzyme coincided with increasing amylose levels. Nevertheless, the possibility of

a preferential transfer of glucose from UDPG (rather than from ADPG as in rice) to amylopectin cannot be excluded.

Starch synthesised from UDPG was less susceptible to β amylolysis than that formed from GIP (Table 11). It may be, therefore, that phosphorylase synthesises de novo only straight chain glucans, whereas both amylopectin and amylose can be synthesised by starch synthetase. Both enzymes appear capable of utilising branched primers more effectively than straight chain ones. Hanes[19] noted that starch synthesised by phosphorylase resembled amylose in that it contained no branch points. α-1,6 links have been detected in the products of starch synthetase.[81] De Fekete[74] observed that starch synthesised from GIP could be solubilised by phosphorylase, but not starch synthesised from ADPG, and concluded that the pathways of starch formation by these two enzymes were different. Since it is uncertain that starch synthetase itself can form α-1,6 bonds, a coupled reaction could occur between this enzyme and branching enzyme which does not occur with phosphorylase. The two enzymes may be related spatially, as has been suggested for glycogen synthetase and branching enzyme in rat liver.[88]

4.4. Synthesis of ADPG and UDPG

It has already been noted that while sucrose may be converted to UDPG by sucrose-UDP glucosyl transferase, it is improbable that significant amounts of ADPG are synthesised by this route.

Both UDPG- and ADPG-pyrophosphorylase were detected in barley endosperm although at somewhat lower levels of activity than recorded in maize.[53] The relationship of these enzymes to inorganic pyrophosphatase is interesting, since Amir et al.,[89,90] have shown that ADPG production is completely inhibited by pyrophosphate, and suggest that the equilibrium of the reaction is controlled by the pyrophosphate concentration. In barley endosperm, the peak of ADPG pyrophosphorylase activity was found to coincide with the period of increasing pyrophosphatase activity. Thus synthesis rather than pyrophosphorylysis of ADPG would be favoured. UDPG pyrophosphorylase, on the other hand, could be detected before the ADPG enzyme, and was quite active around 15 days, when pyrophosphatase was still low. The peak of activity occurred around 25 days, when pyrophosphatase was also at a maximum, and ADPG activity was declining. This implies a change in the preferred direction of UDPG pyrophosphorylase during grain development. Low pyrophosphatase activity between 12 and 16 days after anthesis would favour pyrophosphorylysis of UDPG to form UTP and GIP. This would allow the conversion of sucrose to GIP, as suggested by Turner and Turner,[68] and supported by de Fekete and Cardini.[32]

$$\underset{\text{(sucrose)}}{G-F} + UDP \rightleftharpoons UDPG + F \quad \text{(sucrose-UDP glucosyl transferase)}$$

$$UDPG + PP_i \rightleftharpoons UTP + GIP \quad \text{(UDPG pyrophosphorylase)}$$

The GIP formed could be immediately polymerised to form α-1,4-glucans by phosphorylase activity. However, the concomitant increase in pyrophosphatase, ADPG pyrophosphorylase, and ADPG-starch synthetase would allow incorporation of glucose into starch via ADPG.

$$GIP + ATP \rightleftharpoons ADPG + PP_i \quad \text{(ADPG pyrophosphorylase)}$$

$$(PP_i \longrightarrow 2\ P_i) \quad \text{(inorganic pyrophosphatase)}$$

$$ADPG + (G)_n \longrightarrow ADP + (G_{n+1}) \quad \text{(starch synthetase)}$$

Increasing pyrophosphatase activity would also favour UDPG synthesis rather than degradation, so that both UDPG and ADPG could be synthesised by this reaction and incorporated into starch. However, the activity of sucrose-UDP glucosyl transferase exceeds that of UDPG pyrophosphorylase during this period and so it is likely that further synthesis of UDPG by the pyrophospherylase pathway would be inhibited by high UDPG concentrations. Moreover, there would be competition between UDPG and ADPG enzymes for available GIP. Coupling of UDPG pyrophosphorylase (in the direction of UDPG breakdown) and ADPG enzyme (in the direction of ADPG synthesis) would ensure rapid conversion of sucrose to GIP. A similar scheme was suggested in maize endosperm by De Fekete and Cardini.[32]

$$\text{Sucrose} \longrightarrow UDPG \underset{+\ PP_i}{\rightleftharpoons} UTP + GIP$$

$$\uparrow\ +\ PP_i$$

$$\text{Starch} \longleftarrow ADPG \rightleftharpoons ATP + GIP$$

The detection of nucleoside diphosphate kinase in soluble endosperm extracts around this period indicates that UTP formed from UDPG may be utilised to reform ATP required for further ADPG synthesis.

$$UTP + ADP \rightleftharpoons UDP + ATP$$

Evidence for a similar mechanism was also detected in maize endosperm.[32]

A pathway for the formation of GIP from fructose has been proposed by many workers.[32,74,91] Hexokinase, glucose-6-phosphate ketoisomerase, and phosphoglucomutase were all detected in soluble endosperm extracts from about 3 days after anthesis. Hexokinase activity showed increases in rate as early as 8 days, followed by rising glucose-6-phosphate ketoisomerase and phosphoglucomutase around 14 days after anthesis. Patterns of activity and maximum reaction rates achieved resembled those observed in maize,[53] although no bound form of phosphoglucomutase was detected in barley endosperm. Thus fructose could be converted to GIP and subsequently utilised in starch synthesis by the following sequence of reactions.

$$F + ATP \longrightarrow F6P + ADP$$

$$F6P \rightleftharpoons G6P$$

$$G6P \rightleftharpoons GIP$$

Thus both hexose moieties of sucrose would be incorporated into starch.

It is of interest that UDPG-linked starch synthetase could be detected in the amyloplast fraction of barley endosperm before either UDPG-pyrophosphorylase or sucrose-UDP

glucosyl transferase showed detectable activity in the soluble fraction. Amyloplasts of 5 to 7 day endosperms could also be stained with iodine reagent. Bearing in mind the requirement of transglucosylases for a glucan primer, it appears likely that a separate mechanism of starch synthesis may operate in young endosperms.

4.5. Phosphorylase

It has been reported that certain phosphorylases are capable of synthesising an amylose-like glucan in the absence of added primer.[36,37,92,93]

In barley endosperm, phosphorylase could be detected several days before starch synthetase, although the periods of maximum activity of the two enzymes coincided. A similar pattern of activity could be detected in the absence of added primer. When unprimed activity was considered as a percentage of total phosphorylase it became evident that the most important phase occurred between 2 and 10 days after anthesis, when unprimed phosphorylase constituted 70-80 percent of the total activity. The proportion of unprimed activity fell sharply as starch synthetase increased. A secondary peak in the proportion of unprimed activity appeared around 22 days; this was probably due to the accumulation in the soluble endosperm fraction of short chain oligosaccharides, which were nevertheless sufficient to prime phosphorylase activity. Bailey et al.,[94] considered maltotriose to be the smallest maltosaccharide capable of priming phosphorylase,

although maltotetraose was four times as efficient. On the other hand Green and Stumpf[95] found that decyclised Schardinger dextrins (containing up to six glucose residues) would not prime potato phosphorylase. In general however, it seems certain that starch phosphorylase can utilise much shorter chain primers than either muscle phosphorylase or starch synthetase.[96] Consequently the possibility that so-called 'unprimed' phosphorylase activity is due to priming by endogenous maltosaccharides or by carbohydrate contamination of GIP must always be considered. This will be discussed in more detail later.

Since phosphorylase may catalyse both synthesis and degradation of starch, the inorganic phosphate/labile phosphate ratio in the micro environment of the enzyme provides an important control mechanism.[18,19] Measurements of P_i and labile phosphate concentrations in barley endosperm indicated that the overall P /labile phosphate ratio was closely related to the proportion of unprimed phosphorylase activity, though no labile phosphate could be detected before 10 to 11 days after anthesis. Possibly synthesis of starch is favoured in young endosperm and degradation in older grain. The exact significance of these measurements is however, open to debate, since they only reflect phosphate concentrations in the endosperm as a whole, and not in the micro environment of the enzyme protein.

Although phosphorylase-catalysed starch synthesis was progressively inhibited by increasing concentrations of inorganic phosphate, the extent of inhibition varied, both

with the age of the grain, and with the presence or absence of primer. This suggested that two or more phosphorylase isoenzymes could be present in the barley endosperm during development, having different primer requirements, and exhibiting different susceptibilities to inhibition by phosphate.

Similar indications were drawn from the investigation of pH dependence of phosphorylase activity in the direction of starch synthesis. Enzyme from 7 and 14 day extracts both showed a broad spectrum of pH dependence between 6 and 7 pH units, suggesting a mixture of proteins. With 22 day extracts the sharper peak of primed activity at pH 7.2 indicated a single protein. It has already been noted that the occurrence of 'unprimed' activity in endosperm at this stage is almost certainly due to contamination by endogenous primers.

These results may also be explained in terms of aggregated and dissociated forms of a single enzyme, as in the case of muscle phosphorylase, which exists in the form of a dimer (phosphorylase b) and a tetramer (phosphorylase a). Phosphorylase b, which is active only in the presence of AMP, can be converted to the 'a' form (which is independent of AMP) by phosphorylation of the enzyme protein with ATP and phosphorylase b kinase.

In barley endosperm, however, both ATP and AMP had a slight inhibitory effect on both primed and unprimed activity. Green and Stumpf[95] showed that adenylic acid was not a component of purified potato phosphorylase, and

had no activating effect on the enzyme. Inhibition of maize phosphorylase isoenzymes by ATP was also observed by Tsai and Nelson.[37] It seems clear that starch phosphorylase in higher plants does not consist of subunits activated by adenosine nucleotides in the same way as the animal system.

4.6. Separation of Phosphorylase Isoenzymes

Electrophoresis of soluble endosperm extracts gave support to a hypothesis of changing patterns of phosphorylase isoenzymes during grain development. Two bands of activity were obtained with extracts from 7, 12-13, and 14-15 day endosperm, but only one main band with 22 day grain. It is unlikely that the slowmoving band was due to the formation of a glycogen-phosphorylase complex since two similar bands could also be detected with 7 and 12-13 day endosperm using glycogen free gels. Unprimed activity was associated mainly with the slow band of 7 day extracts, with both bands of 12 day extracts, and with the fast band of 14-15 day extracts. The absence of unprimed activity in 22 day extracts supported the proposal that the unprimed activity previously noted in extracts of this age was due to soluble endogenous primers, Since these would be separated from the enzyme by electrophoresis.

This pattern of electrophoretic zones suggests that two phosphorylase isoenzymes persist in young barley endosperm up to 15-16 days after anthesis; after this stage one

is degraded. It would appear that unprimed activity is associated exclusively with the slow moving isoenzyme of young endosperm but as development proceeds the potential for unprimed activity is progressively acquired by the second isoenzyme. This evidence does not, however, differentiate between the amount of enzyme present and its activity and it is likely that the change in location of unprimed activity reflects the amount of each isoenzyme present. Thus in 7 day endosperm the slow moving component which is capable of unprimed activity, predominates; this isoenzyme gradually disappears as the second component accumulates. Initially the second isoenzyme is also capable of unprimed activity, but this potentiality is soon lost, so that by 22 days after anthesis only one isoenzyme, requiring a glucan primer, persists. The loss of unprimed activity may reflect modification of the isoenzyme without alteration of characteristic electrophoretic migration. This is borne out by the results of column chromatography of 22 day extracts.

Two electrophoretic zones of phosphorylase activity were detected by De Fekete[74] in extracts from spinach leaves and immature cotyledons of Vicia faba, and also in immature barley endosperm.[97] Gerbrandy and Doorgeest,[98] who separated several phosphorylase isoenzymes from potatoes, found that different isoenzyme patterns appeared during periods of starch synthesis and starch degradation.

They suggested that certain isoenzymes were active mainly in the direction of synthesis, while others were concerned with degradation.

If phosphorylase is actively synthesising α-1,4-glucans in young endosperm, either de novo or using small maltosaccharide precursors, the reaction products would be available as primers for starch synthetase. Thus incubations of GIP and UDPG with soluble endosperm extract should show incorporation of glucose from both glucosyl donors into starch. In barley endosperm incorporation of ^{14}C-glucose from G*IP could be detected as early as 2-3 days after anthesis, and this activity was accelerated by the presence of UDPG. By 5 days after anthesis, UDPG had an inhibitory effect on glucose incorporation from G*IP, this effect being especially pronounced over longer incubation periods. ADPG and UDPG have been shown to inhibit starch phosphorylase[74] and glycogen phosphorylase in animals.[99] Since this inhibition was not evident in extracts from 2-3 day and 16 day barley endosperm, the effect may be due to competition between starch synthetase and phosphorylase for available primer. Assuming that 2-3 day endosperms contain no endogenous primer, starch synthesis by phosphorylase alone will be extremely slow, thus addition of UDPG (allowing labelled glucans to be elongated by starch synthetase) will cause a significant increase in the overall incorporation of ^{14}C-glucose from G*IP into starch. 5-7 day extracts, on the other hand, will already contain small endogenous maltosaccharides as a result of phosphorylase activity in vivo. If these are also sufficient to prime starch synthetase there will be competition between the two enzymes for available primer, which at this stage will be severely limited. Thus incorporation from G*IP will be reduced when

both G*IP and unlabelled UDPG are present. By 16 days after anthesis, primer will no longer be limiting, and addition of UDPG will not affect incorporation from G*IP.

Similarly, incorporation from UDPG* will be reduced by the presence of GIP in 5-7 day extracts.

These results contrast with those of Bird,[38] who obtained enhanced incorporation when both UDPG and GIP were present in incubations with chloroplast extracts. However, these extracts were obtained from destarched chloroplasts, which presumably contained no endogenous primer, corresponding to the situation obtained with 2 to 3 day barley endosperm. In some cases where barley ears were allowed to stand in water overnight before extracts were prepared, a similar enhancement of glucose incorporation was observed when both UDPG and GIP were added. Presumably endogenous primer is used up by respiration in this case.

(It has already been noted that starch synthetase could not be detected in amyloplast fractions before 5 days after anthesis. However at 7 days after anthesis, soluble activity exceeded amyloplast-bound activity, and it is therefore probable that all starch synthetase is soluble in the very young endosperm. It must also be remembered that starch synthetase activity was measured during an incubation time of 4 hours only, whereas incubation times were extended to 12 and 24 hours for glucose incorporation experiments.)

The possibility that unprimed phosphorylase activity is due to carbohydrate contamination of GIP has already been mentioned. Green and Stumpf[95] could detect no unprimed

activity by potato phosphorylase using purified GIP. Kamogawa et al.,[100] also purified GIP extensively by charcoal treatment. A longer lag phase of potato phosphorylase was observed using this GIP, but unprimed activity was not abolished and final rates of activity were similar to those obtained with unpurified GIP. Muscle phosphorylase, on the other hand, was inactive in the absence of primer using purified GIP. Bird[38] detected similar rates of unprimed phosphorylase activity in chloroplast extracts using GIP purified by three different methods of unpurified GIP. With barley endosperm extracts, incorporation of glucose into starch could be detected using only chromatographically pure G*IP as substrate. It would therefore seem evident that contamination of GIP is not significant.

Contamination by endogenous primers remains probable, since a soluble endosperm extract was used as enzyme source for most experiments. Prior treatment of the extract with glucoamylase caused a considerable increase in both primed and unprimed phosphorylase activity. The increase in primed activity is obviously due to glucoamylase attack on the starch primer, giving rise to more small glucan chains. The increase in unprimed activity suggested that the endosperm extract contained a limited amount of soluble α-1,4-glucans (maltosaccharides) which could prime the reaction, but which could be broken down by glucoamylase to provide a larger number of suitable primer chains. This increase in activity could be considerably reduced, but not abolished, by dialysis, which would remove hexoses and smaller maltosaccharides such

as maltose, maltotriose and maltotetraose. Presumably some primer molecules released by glucoamylase activity were too large to be lost by dialysis, or were protein bound. Similar activation of unprimed phosphorylase by glucoamylase was observed after partial purification of the endosperm extract by ammonium sulphate precipitation. (Although no unprimed activity could be detected in untreated fractions over a period of two hours, incorporation of glucose from GIP was obtained with similar extracts during 24 hour incubations). It seems evident, therefore, that barley endosperm contains some form of carbohydrate primer which is protein bound, and is precipitated with the enzyme protein by ammonium sulphate treatment.

It is perhaps surprising that no corresponding activation of phosphorylase occurred after β amylase treatment, even with undialysed mixtures. Fuwa[83] notes that phosphorylase in maize kernels is activated by α amylase but inhibited by β amylase. Possibly all endogenous maltosaccharides were rapidly broken down to maltose and maltotriose, but in that case one would expect considerable variation between dialysed and undialysed extracts. In fact no significant difference was observed. An alternative explanation is that the endogenous maltosaccharides were too small to be greatly affected by β amylase during the two hour incubation period, although Bailey suggests that maltotetraose is the smallest possible substrate for this enzyme.[94]

The partial purification of barley endosperm phosphorylase by column chromatography supported indications from electrophoresis experiments that young endosperms (up to 15

days after anthesis) contain two isoenzymes, but older grain only one isoenzyme. This behaved differently on chromatographic separation, and may represent a modification of one of the preexisting isoenzymes, as previously mentioned. Unprimed activity appeared to be associated mainly with the first fraction to be eluted from the column (this may correspond to the slower migrating electrophoretic zone) and was most evident in extracts from young endosperm. Tsai and Nelson[37] also separated two isoenzymes of phosphorylase from developing maize endosperm by DEAE-cellulose chromatography, one of which was capable of synthesising starch in a 'primer-free' system. This isoenzyme, however, could not be detected before 12 days after anthesis. The second isoenzyme, which was found in young endosperm, required a long chain glucan primer. It therefore seemed doubtful that either isoenzyme could be responsible for primer synthesis in maize endosperm. Since incubation periods were only 30 and 45 minutes in duration it may be possible that low levels of unprimed activity in young endosperm were not detected.

Maintenance of unprimed activity throughout the purification procedure reduces the possibility of its being attributable entirely to contamination by endogenous maltosaccharides. Rather, it is probable that a primer of a maltosaccharide nature could be covalently bound to the enzyme protein. No carbohydrate could be detected in any fractions eluted from the DEAE-cellulose column, but it is quite possible that concentrations of such a bound primer would be too small to be detected by the method used. Fukui

and Kamogawa[101] found that a purified crystalline preparation of potato phosphorylase contained approximately 0.2 to 0.6 glucose residues/mole enzyme protein. Removal of this carbohydrate by glucoamylase treatment abolished unprimed activity. Fredrick[93] obtained similar results with phosphorylase isoenzymes from algae, and suggested that the enzymes were glycoproteins.

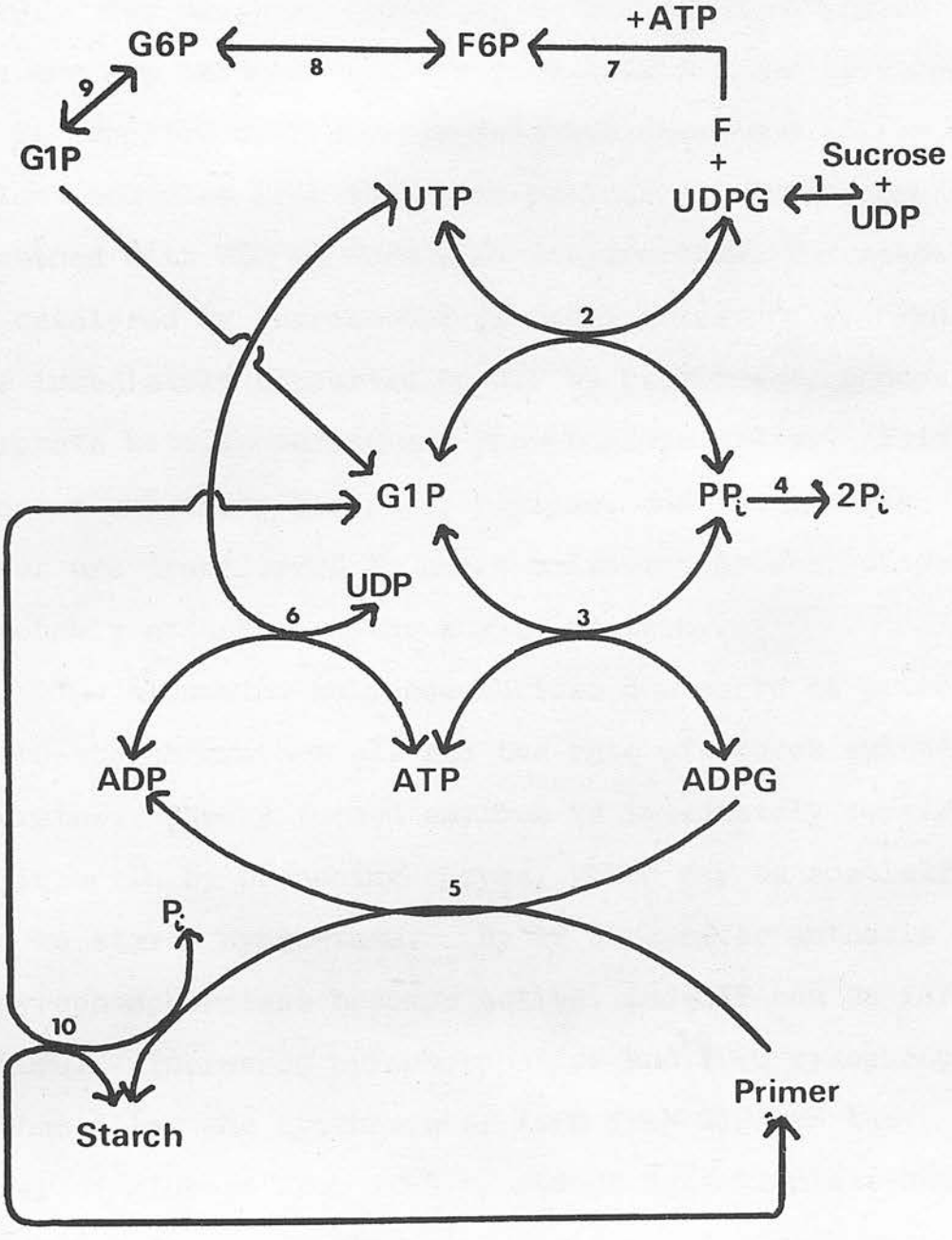
Indications of a similar protein-bound glycogen primer have also been observed in animal systems. Krisman[102] reported that a partially purified glycogen synthetase preparation from rat liver catalysed the transfer of glucose from UDPG to a TCA-insoluble fraction. She suggested that this contained a protein-bound α-1,4-glucan. Wanson and Drochmans[103] noted that the last 3% of a contaminating protein could not be removed from a pure preparation of native glycogen.

The results of the present investigation are consistent with the appearance in the barley endosperm soon after anthesis of two isoenzymes of phosphorylase, possibly both glycoproteins. Around 7 days after anthesis unprimed activity is predominantly associated with the slow electrophoretic zone and with the first active fraction eluted from the DEAE-cellulose column. This isoenzyme gradually loses its capacity for unprimed activity, which is transferred to the second isoenzyme. Around 15 days after anthesis the first isoenzyme disappears. The second isoenzyme also loses its unprimed activity (possibly by removal of the carbohydrate moiety), this modification increasing its rate of elution from the column, but not affecting its electrophoretic mobility.

It has also been suggested that starch synthetase may exist as different isoenzymes, some of which are capable of synthesising starch in an unprimed system.[79,80,81] Ozbun et al.,[81] separated two isoenzymes of soluble starch synthetase from waxy maize, and obtained unprimed starch synthesis with fraction 1. α-1,6 links in the starch synthesised in the unprimed reaction indicated contaminating branching enzyme. Tanaka and Akazawa,[79] working with waxy and non-waxy rice seeds, also separated two active fractions of starch synthetase, one of which (fraction 2) utilised short chain maltosaccharide primers more readily than the fraction 1. All fractions were contaminated with amylase activity. Since both rice and maize extracts were prepared from seeds at the milky stage of development (about 22 days after anthesis), these results provide no evidence that such isoenzymes could contribute to primer synthesis in very young endosperm.

Figure 29. Proposed scheme of starch synthesis in barley endosperm. (branching enzyme and UDP-starch synthetase omitted)

1. Sucrose-UDP glucosyl transferase
2. UDPG pyrophosphorylase
3. ADPG pyrophosphorylase
4. Inorganic pyrophosphatase
5. ADPG-starch synthetase
6. Nucleoside diphosphate kinase
7. Hexokinase
8. Glucose-6-phosphate keto-isomerase
9. Phosphoglucomutase
10. Phosphorylase


G6P
F6P
+ATP
G1P
F
+
UDPG
Sucrose
+
UDP
UTP
G1P
PPi
2Pi
UDP
ADP
ATP
ADPG
Pi
Starch
Primer
1
2
3
4
5
6
7
8
9
10

4.7. Integrated scheme of starch synthesis

The proposed scheme by which sucrose is converted to starch in developing barley endosperm is depicted in Fig. 29. For clarity, branching enzyme and UDPG starch synthetase are omitted. After fertilisation the developing grain is supplied with sucrose from the stems and leaves of the plant and also from the testa-pericarp. This sucrose is combined with UDP to form UDPG and fructose, the reaction being catalysed by sucrose-UDP glucosyl transferase. Fructose can be immediately converted to GIP by hexokinase, glucose-6-phosphate keto isomerase and phosphoglucomutase. This provides a substrate for phosphorylase, and the glucose residues are transferred to short maltosaccharides, which are probably attached to the enzyme protein.

The elongated maltosaccharides now serve as primers for UDPG-starch synthetase, and the rate of starch synthesis accelerates. Newly formed amylose is immediately converted to amylopectin by branching enzyme, which may be spatially linked to starch synthetase. By 12 days after anthesis UDPG-pyrophosphorylase becomes active, and GIP can be formed from UDPG. Increased pyrophosphatase and ADPG pyrophosphorylase then allow the synthesis of ADPG from GIP and the transfer of glucose from ADPG to starch by amyloplast-bound starch synthetase; a rapid proliferation of starch ensues, allowing the accumulation of amylose.

The carbohydrate-containing isoenzymes of phosphorylase now begin to disappear, and the synthetic activity of the enzyme is restricted, owing to the decreased availability

of GIP and increasing phosphate concentrations. Degradative phosphorylase attack on newly formed glucans, together with amylolytic activity, will further accelerate the process of starch accumulation by providing more primer molecules.

Two important factors must also be considered in any proposed scheme of starch synthesis:-

1. The relevance of enzyme activities determined in tissue homogenates to those prevailing under in vivo conditions.
2. The relationship between the amyloplast and soluble fraction of the endosperm.

1. Homogenisation of any tissue will destroy much of the internal organisation of the tissue and cellular structure, and bring about the dilution of substances such as phosphates and key intermediates which may well have existed in totally different concentrations at the site of starch synthesis. This must be carefully considered when results obtained in vitro are applied to events occurring in the living plant.

2. Since the amyloplast is a membrane-bound organelle a boundary will exist between many of the enzymes involved in starch synthesis, and the site of starch accumulation, that is, within the amyloplast membrane. While most of the enzymes investigated in the present study were located in the soluble extract of the endosperm, branching enzyme, starch synthetase and some phosphorylase activity could also be detected in the amyloplast fraction. This fraction was not purified, and could contain contaminating nuclear material,

as well as mitochondria and protein bodies. However since the relevant enzymes are all closely concerned with starch synthesis or degradation, it is most probable that they would be associated with the amyloplasts rather than with other organelles. A difference in the extent of attachment of these enzymes was immediately apparent. Phosphorylase activity could be removed by washing with buffer, which did not affect either starch synthetase or branching enzyme. Starch synthetase was highly active in intact amyloplast preparations, but appreciable branching enzyme activity could only be detected after mechanical disruption of the amyloplasts. This argues strongly for a fixed spatial arrangement of these enzymes with respect to the amyloplast. Thus in the young grain phosphorylase and branching enzyme could be loosely associated with the amyloplasts. Although most of the starch synthetase detected in young endosperm is soluble, some may be attached to the outer membrane of the amyloplasts, which contain very little starch at this stage. A glucan synthetase (UDP glucose: glucose glucosyl transferase) has been detected in the plasma membrane fractions of onion stem extracts.[104]

It is proposed that short chain maltosaccharides are synthesised by phosphorylase activity, then elongated and α-1,6 links introduced by starch synthetase and branching enzyme. These enzymes would become trapped by deposition of newly synthesised starch around the periphery of the amyloplast. Thus starch synthetase activity detected in the amyloplast fraction of the endosperm during the period

of starch accumulation would include not only enzyme attached to the amyloplast membrane, but also starch-bound enzyme. This latter probably corresponds to the bound starch synthetases previously referred to (see, for example, 22, 35, 86) which were prepared from acetone powders of starch granules from various plant sources. The formation of an enzyme-polysaccharide complex modifies the specificity of starch synthetase with respect to the glucosyl donor, whereas branching enzyme becomes almost completely inactivated after incorporation into the amyloplast. It is possible that branching enzyme requires a certain degree of mobility to connect between donor and acceptor amylose chains, and this would be increasingly limited as the enzyme became surrounded by amylopectin molecules.

Close association of polysaccharide synthesising enzymes has been demonstrated in animal systems.[88] A particulate preparation, termed a 'glycosome', which contained enzymes of glycogen synthesis and degradation as well as branching enzyme activity, could be separated from rat liver extracts by precipitation of the glycogen-enzyme complex.

Amyloplasts from several plant sources contain more than one starch grain. Barley endosperm amyloplasts contain only one large starch grain, but around 20 days after anthesis small granules have been observed in the plastid stroma which were released into the cytoplasm by extrusion of the amyloplast membrane (page 2,[4,5]). The initiation of these secondary grains is unclear. Badenhuizen[105] has suggested that the internal membranes of the amyloplast may

form 'pockets' in which starch precursors and enzymes could accumulate. This would allow the formation of small starch granules around the periphery of the plastid stroma. It is difficult to see how such a model could explain the formation of a single starch grain, unless the internal divisions were evenly arranged. The polarization cross which is visible when barley amyloplasts are viewed under plane polarised light indicates a regular structure, possibly due to deposition of starch in concentric shells. However, such a structure would be consistent with a concerted synthesis of starch over the entire surface of the amyloplast by soluble and membrane-located starch synthetase, together with phosphorylase and branching enzyme, as outlined above.

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SHORT COMMUNICATION

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Abstract—The activity of starch synthetase in amyloplasts from barley endosperm was measured. The relative effectiveness of UDPG and ADPG as glucosyl donors was determined, and correlated with amyloplast age. Only UDPG linked synthesis could be detected up to about 12 days after anthesis. After this time ADPG was the main glucosyl donor.

INTRODUCTION

THE INVOLVEMENT of sugar nucleotides in starch synthesis was first reported by De Fekete *et al.*[1] Since then both UDP and ADP[2, 3] linked mechanisms have been observed in many plant tissues. There is some evidence that the rate of glucose transfer from ADPG may exceed that from UDPG.[3] This paper describes an investigation into the relative importance in the developing amyloplasts of UDP glucose α1–4 glucan α4 glucosyl transferase (E.C. 2.4.111 starch synthetase) and the corresponding activity using ADPG as glucosyl donor.

RESULTS

Starch was visible in the plastids from 2 to 3 days after anthesis, although starch synthetase activity was detected only from 6 days after anthesis. Plastid size increased until 25 days. From 21 days onward small plastids could be seen inside the larger ones.[4] No starch was detected in the soluble fraction.

Both UDPG and ADPG linked starch synthetase increased in activity with plastid size (Fig. 1). However, the relative activity of the UDPG enzyme decreased with increasing plastid size (Fig. 2) whilst the ADPG enzyme became increasingly important. When starch was added to the assay system there was an increase in activity in the plastids. The percentage increase was greatest with very young plastids (about 6–7 days after anthesis) indicating that at this stage of development primer concentration is limiting.

The starch synthetase activity of the soluble fraction at first appeared to be greater than that of plastids of the same age. The activity of the soluble fraction of 14 day grains exceeded that of plastids of the same age by a factor of 10^3 (using UDPG) or 10^2 (using ADPG), when assayed by UDP (or ADP) production. There was, however, no stimulation of activity when starch was added as a primer, although the original soluble fraction did not contain any

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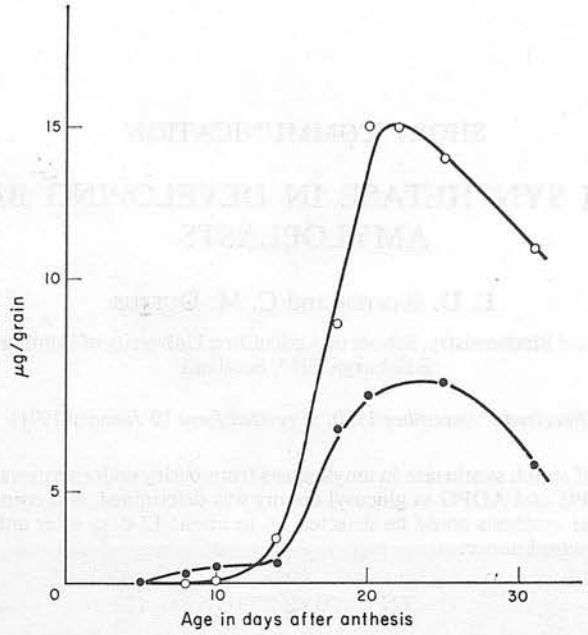

FIG. 1. LEVELS OF NUCLEOSIDE PYROPHOSPHATES SYNTHESIZED BY PLASTIDS DURING 2 hr INCUBATION IN μgm/grain.

ADP and UDP values corrected for endogenous levels (see text) ● UDP; ○ ADP.

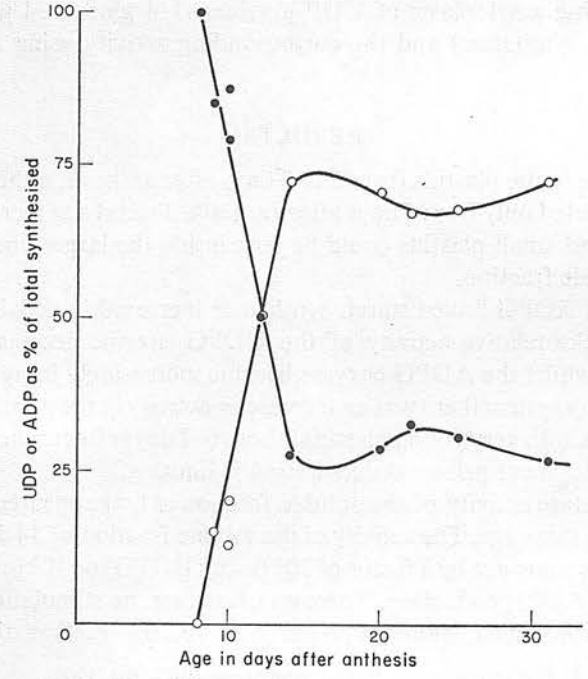

FIG. 2. AMOUNT OF UDP (OR ADP) SYNTHESIZED BY PLASTIDS EXPRESSED AS A PERCENTAGE OF TOTAL UDP + ADP SYNTHESIZED.

● UDP; ○ ADP.

detectable starch. Enzyme fractions were therefore incubated with UDP (^{14}C) G and starch as a carrier. Incorporation of (^{14}C) glucose into an insoluble product associated with the starch pellet occurred only with the plastid fraction. This suggests that the high concentrations of UDP and ADP detected in the previous assays of the soluble fraction are formed by the breakdown of the UDPG and ADPG added without the glucosyl moiety being incorporated into a high molecular weight polymer. These high levels of UDP and ADP may be due to the activity of sucrose synthetase, an enzyme which is generally reversible,[5] although synthesis of sucrose may be somewhat slower than the reverse reaction at physiological pH.[6] This enzyme is highly reactive in the soluble fraction of barley grain homogenates. The specific activity (in nmoles sucrose/min/grain) increased from 1·4 at 7 days after anthesis to a peak of 624 at 28 days, afterwards levelling off to a value of around 500 in the mature grain. Similar, or slightly lower, levels of activity in the direction of sucrose synthesis, could account for all the UDP and ADP produced by the soluble fraction during the assay for starch synthetase by the pyruvate kinase method. No sucrose synthetase activity could be detected in the plastids.

DISCUSSION

These results agree with those of previous workers[3, 7, 8] which suggest that proliferation of starch in developing seeds is mainly brought about by ADPG linked starch synthetase associated with the amyloplasts. Starch is, however, visible in the plastids before starch synthetase activity can be detected. Also, this enzyme has been shown to require an oligosaccharide primer.[2] These observations suggest that a different enzyme is involved in the early synthesis of starch from low molecular weight primers. Tsai and Nelson[9] have isolated four phosphorylase isoenzymes from maize, three of which are present in the developing endosperm and one in the embryo. Their work suggests that two of the endosperm isoenzymes may be capable of synthesizing starch without the addition of primer. Slabnik and Frydman[10] have detected a similar phosphorylase enzyme in potatoes. Classical phosphorylase activity can be detected in the soluble fraction of young barley endosperm, using soluble starch as a primer. This activity increased from 195 ng starch produced/grain/min at 12 days after anthesis to 334 ng at 19 days. So far attempts to obtain synthesis of starch from glucose-1-phosphate by phosphorylase without added primer have been unsuccessful. However, there is some evidence[9] that an inhibitor of certain phosphorylase isoenzymes may be present in crude cereal grain homogenates. Bird[11] has shown that in destarched chloroplasts supplied with glucose-1-phosphate, phosphorylase will bring about the synthesis of an insoluble glucose polymer which is sufficient to prime the starch synthetase reaction. In young barley endosperm, such a primer would be slowly converted into starch by UDPG linked starch synthetase. By 12–15 days after anthesis, when ADPG linked starch synthetase has become more active, the starch content of the plastids increases rapidly and starch is no longer limiting. At this later stage UDP and UDPG may be involved in the utilisation, via sucrose synthetase, of the sucrose supplied to the endosperm by the leaves.[5, 6]

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EXPERIMENTAL

Plant material. Two row barley plants (*Hordeum distichium* (L.). Lam. v. Maris Baldric) were used. Conditions of growth and methods used to determine the date of anthesis were as described by Merritt and Walker.[12] Grains could be stored at −15° for 3 months without loss of activity.

Preparation of amyloplasts. Endosperms were separated from embryo and aleurone by hand, homogenised in an all-glass Potter type homogenizer in 2 vol. of H_2O and filtered through double muslin to remove cell debris. Amyloplasts were separated from the soluble components by centrifugation for 10 min at 4° and 2500 *g*. They were resuspended in H_2O.

Enzyme assays. Starch synthetase was assayed by the method of Leloir,[2] but in a total volume of 0·4 ml using 0·1 ml of the suspended amyloplasts as the source of enzyme. The supernatant solution from the centrifugation, containing the soluble components of the endosperm, was also assayed for synthetic activity. All assays were corrected for endogenous nucleotides using controls incubated without UDPG or ADPG.

ADP and UDP were measured by the pyruvate kinase method[13] and compared with standards run simultaneously. Assays carried out without amyloplasts showed that neither UDPG nor ADPG dissociated under the conditions of the assays. Starch synthetase assays were also carried out using ^{14}C labelled UDPG (see Leloir).[2] UDP-(^{14}C) G (ammonium salt, 237 mc/mM) was obtained from the Radiochemical Centre, Amersham. The soluble fraction was assayed for phosphorylase by the method of Slabnik and Frydmann.[10] Sucrose synthetase was assayed by the method of Avigad and Milner.[14]

Light microscopy. Each amyloplast preparation was examined using a light microscope equipped with phase contrast lenses (magnification ×500). An age approximating to the number of days after anthesis was assigned to each preparation according to the average plastid size. The presence of starch was detected by staining with a 2% KI–0·2% I_2 solution.

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